Effect of Solid Lipid Nanoparticle-Encapsulated Antimicrobial Peptide on Keratinocyte Migration and Wound Healing

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DEDICATION

I would like to thank my family, who has stood with me throughout the process of my Master's Thesis and residency. In particular, my father who has aided in many editing sessions and my mother who has listened to me complain about my long list of challenges. My parents have been my largest critics and have always stressed that I should continue to constantly strive to better myself. Thank you for the countless years you have invested in me and for not giving up on me when I was not at my finest. You have been my fortitude through the long journey of my life and I would never have made it to where I am today without your help. I would also like to thank my girlfriend Rachel who has helped me maintain my sanity during the past two years. It was the small things you did which made all the difference. The many meals you made for me, helping around the house and giving me extra time to work on projects. I will never forget those things you have done for me that made my life easier. I hope one day I can repay you when you venture into similar life challenges.

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ABSTRACT

Objective: This study evaluated the effect of various drug delivery formulations on human keratinocyte migration and viability following treatment with the synthetic antimicrobial peptide KSL-W alone, KSL-W encapsulated in solid lipid nanoparticle (KSL-W + SLN), or solid lipid nanoparticles (SLN) alone evaluated by electric cell-substrate impedance sensing (ECIS) (Applied Biophysics), photographic analysis and cell viability. **Methods**: HaCaT cells were seeded on an 8W10E ECIS plate at a concentration of 3.125 $x10^5$ cells/ml in high glucose Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% Streptomycin/Amphotericin B for 28 hrs with a medium change at 12 hrs for fetal bovine serum free medium. Wells were treated at 27.5 hrs with a positive control [2ng/ml transforming growth factor β1 (TGF-β1)], negative control (2 µg/ml somatostatin), 200 µg/ml KSL-W, 200µg/ml KSL-W + SLN, or SLN. Cells were wounded at 28hrs with 1600mW electrical pulse for 20 seconds. Impedance was measured at treatment, prior to wounding and 1 hr, 5 hrs, and 24 hrs post wounding. A second assay was performed with 1 x 10^5 cells incubated for 48hrs. Media was changed and supplemented with either 2 ng/ml TGF-β1, 2 µg/ml somatostatin, 200 µg/ml KSL-W, 200 µg/ml KSL-W + SLN, or SLN. Cells were then allowed to incubate for an additional 12 hrs, isolated and counted for viability. Pre- and post-treated cells were analyzed by 5x inverted confocal light microscopy. All experiments were completed in triplicate. One-way ANOVAs were performed, followed by Tukey pairwise comparisons. Data was analyzed using Minitab 16 and Statsplus. P-values greater than 0.05 were considered significant. **Results:** Treatment with formulations of 200 µg/ml KSL-W,

200 μg/ml KSL-W + SLN and SLN demonstrated no beneficial effect on migration of HaCaT cells when compared to untreated cells and cells treated with 2 ng/ml TGF-β1. Significant differences were found between groups of control, positive control and negative control when compared to KSL-W, KSL-W +SLN and SLN ($p<0.01$), but no significant difference within the group of KSL-W, KSL-W +SLN and SLN ($p=0.523$). The different formulations (KSL-W, KSL-W+SLN and SLN) showed a significant reduction in the change in impedance of HaCaT cells. There was no significant difference found between negative control (somatostatin) and untreated cells when measured by ECIS. Significant differences in cell viability were seen between treatment groups KSL-W and negative control ($p= <0.01$) when compared treatment groups SLN and KSL-W + SLN. Through not statistically different for all groups, SLN and KSL-W + SLN show much less viability when compared to all other treatment groups. **Conclusions:** The proposed formulation of SLN encapsulated KSL-W appears to decrease migration when compared to untreated cells when measured by ECIS. The SLN encapsulation may additionally affect cell viability of keratinocytes that are grown on polystyrene substructure without fibroblasts. This decrease in migration and cell viability may make it a poor formulation for treatment of microbes. Additionally, somatostatin, at 2 μg/ml does not appear to act as a significant inhibitor of the migration of keratinocytes and thus should not be used at the current concentration as a negative control for future experiments. Additional studies need to be completed to validate the results when compared to other drug carriers for KSL-W and experimental designs.

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I. BACKGROUND AND LITERATURE REVIEW

A. Keratinocytes and Wound Healing

Epithelial wound healing involves three highly regulated overlapping phases: inflammation, epithelialization and tissue remodeling (Secor, 2011). Wounds, which have been colonized by bacterial biofilms, display abnormal progression through these phases and typically demonstrate prolonged inflammation and failure to re-epithelialize (Secor, 2011). Human keratinocytes, which are the most abundant cell type in the epidermis, are essential for wound healing (Secor, 2011). They act as a key cell in the host's defense, by acting as a barrier to invading bacterial pathogens and microbial components (Miller, 2008) as well as activating the inflammatory cascade (Spiekstra, 2007). In addition, they are directly involved in the innate immunity, secretion of cytokines and the facilitation of migration through alterations in their cell morphology (Raja, 2011).

The migration of keratinocytes has been reported to be influenced by myriad different extracellular hormones in the body (Chernyavsky, 2004). Somatostatin, a regulatory peptide hormone, has been noted in the literature to inhibit the migration of keratinocytes (Vockel, 2011). Conversely, transforming growth factor-β1 (TGF-β1) has shown the opposite effect and been identified to increase the migration rate and differentiation of keratinocytes into a motile phenotype (Räsänen, 2010).

In addition, keratinocyte migration influences the control of multiple genes, some of the most notable being *akap12, egr3,* and *ptgs2* (Busch, 2008). These

mediators serve as important markers of migration and can be observed at the molecular level by reverse transcription polymerase chain reaction (RT-PCR). Since keratinocytes act as the first line of defense, their response at a molecular level can help indicate whether an inflammatory response is actually occurring (O'Toole, 2001). For this study, immortalized cultured human keratinocytes (HaCaT) cells were used. They are a common cell line, used since 1993 for evaluation keratinocytes (Schürer, 1993). Their reliability and high capacity for proliferation *in vitro* made them an ideal cell line for evaluation of cell migration and wound healing (Schoop, 1999).

B. Effects of Biofilms on Wound Healing

There is increasing evidence in the literature that implicates biofilms as a significant cause of a number of infectious processes (Donlan, 2002) accounting for approximately 80% of all microbial infections (Bjarnsholt, 2008). Bacterial biofilms usually predominate the skin as a primarily gram-positive flora (Bowler, 2001), but when injuries occur to tissue, these biofilms have the tendency to adjust from a primarily gram-positive to a gram-negative microflora (Murray, 2006). This maturational shift in biofilm adds to their pathogenicity by changing their environmental conditions and compromising the host's immune response. These pathogenicities include decreases in pH levels, formation of extracellular matrices, increases in bacterial communication and antibiotic resistance (Marsh 2005). As to date, there are many studies on the virulence expression of bacteria, (Yu, 2007;

Andersen, 2010; Sokol, 1987), but little is known about how biofilms composed of bacterial mono-species and mixed-species impair wound healing and ultimately result in tissue loss through persistent infections. With the declining effectiveness of conventional antibiotics and the increases of antibiotic- resistant organisms (Davies, 2003) there has become a need to investigate alternative avenues for antimicrobials than traditional antibiotics.

C. Types Of Antimicrobials

Colonization of bacteria in the oral cavity is inevitable. Certain bacterial species become embedded in a self-produced “glycocalyx” of polysaccharides, referred to as biofilm (Costerton, 1987). The biofilm coating allows bacteria to establish tolerance or resistance to antibiotic therapies and is involved in approximately 60% of the bacterial infections currently treated (Fux, 2005). With the increased number of antibiotic-resistant bacterial strains, new interest in the roles of natural cationic antimicrobial peptides such as alpha- and beta-defensins, cathelicidins, and histatins, as well as synthetic antimicrobial peptides, are being investigated. Cationic antimicrobial peptides have been shown to display a broad range of antimicrobial properties against gram-positive and gram-negative bacteria, as well as fungi and enveloped viruses (Bals, 2000). In addition to their direct antimicrobial activity, endogenous antimicrobial peptides have been characterized as multifunctional molecules, with functions in inflammation, wound repair, and regulation of the innate (McDermott, 2007) and adaptive (Bals, 2000; McDermott, 2007) immune responses.

It has become increasingly apparent that cationic antimicrobial peptides function via alternative mechanisms in response to pathogens, with biological activities contributing to both the recruitment of immune cells and direct inactivation of pathogens (Dürr, 2002). The α- and β-defensins, which are members of the best-studied class of human antimicrobial peptides (Dürr, 2002; Lehrer, 2002) display chemotactic properties for monocytes, T-cells, dendritic cells and mast cells (Dürr, 2002; Lehrer, 2002; Territo, 1989). The neutrophil-derived proline-arginine (PR)-rich antibacterial peptide PR-39 possesses several distinct functional properties, and has been shown to play a role in neutrophil chemotaxis (Huang, 1997) and function (Shi, 1996; Zanetti, 2004).

D. Development of KSL-W

The development of combinatorial libraries as a source for synthetic cationic antimicrobial peptides has gained interest as an alternative approach to overcoming time constraints and effect limitations of designing novel antibacterial and antifungal agents (Hong, 1998). The cationic antimicrobial decapeptide KKVVFKVKFK (KSL), was originally identified from combinatorial libraries with activity against *Candida albicans*, and later demonstrated to display a broad range of activity against bacteria (Hong, 1998). The much smaller antimicrobial KSL, when compared with the naturally *Xenopus*-derived peptide antibiotic magainin II, was shown to have lower minimum inhibitory concentrations (MICs) for *C. albicans* (almost seven times lower), as well as nine tested bacterial species (Concannon, 2003). Stability studies

of KSL and its analogues revealed critical peptide bonds with susceptibility to enzymatic cleavage, Lys^6-Val^7 and Phe^5-Lys^6, to human saliva and simulated gastric fluids, respectively (Na, 2007). The analogue KSL-W (KKVVF**W**VKFK), whereby the sixth residue (lysine) is substituted with tryptophan ("W" = single letter amino acid abbreviation) proved to be most stable in saliva, while still maintaining its antimicrobial activity (Na, 2007).

E. Solid Lipid Nanoparticles

Solid Lipid Nanoparticles (SLNs) are nanocarrier drug delivery platforms that can contain particulates ranging in size from 50 to 1000 nm, and have been demonstrated to be effective in a number of applications (zur Mühlen, 1998). SLNs are mainly composed of solid phase and surfactant lipids formed through emulsification and possess many unique properties, including the capacity to form thin films on skin and increased molecule penetration in cells (Zhang, 2010). These properties make SLNs ideal for the delivery of the cationic peptides such as KSL-W. Recently, SLN encapsulation was shown to be a viable drug delivery strategy for Vitamin A supplements, retinol and retinyl palmitate, with improved cell uptake, as well as a more controlled, slower release rate, relative to their free drug counterparts (Müller, 2000). Finally, SLNs are easy to manufacture and are stable in water or cream (Wissing, 2003) making them excellent candidates for large-scale manufacturing.

F. KSL-W effect on Wound Healing

Recent studies by Semali in 2007 have been completed evaluating the safety of KSL-W to keratinocytes showing that at effective antimicrobial doses of 3-100 µl/ml, KSL-W showed no inhibitory effects on cell adhesion or proliferation. KSL-W in minute amounts has also shown to increase neutrophil chemotaxis in human cell lines (Williams, 2012) and increase wound healing in stressed mice (Williams, 2012). To this point, there have been no studies investigating the effect of KSL-W, in its free and SLN-encapsulated forms, on human cell lines.

II. OBJECTIVES

A. Objective Overview

The goal of this study was to evaluate the cationic antimicrobial decapeptide KSL-W in a nanocarrier delivery system, and determine if there is a multifunctional role on keratinocyte cell function during the migratory process. There is evidence that KSL-W encapsulated in pluronic block copolymer nanocarrier (Pluronic F68) increases the healing of wounds in stressed mice when compared to Pluronic F68 alone (Williams, 2012). To this point, evidence shows that KSL-W has the potential to increase the healing effects of cell migration under one formulation. There is no current published literature that discusses KSL-W under a SLN formulation or on migration and wound healing. This study is intended to provide insight into the effect of SLN encapsulated KSL-W's interaction with keratinocyte migration and wound healing. The knowledge gained will provide potential applications of KSL-W at this formulation when challenged against keratinocytes and their ability to effectively migrate.

B. Specific Hypotheses

This study tested a specific hypothesis as follows:

1) The encapsulation of antimicrobial KSL-W within a solid lipid nanoparticle, the solid lipid nanoparticles by themselves, and KSL-W by itself have an effect on the migration of human keratinocytes when compared to untreated cells

II. MATERIALS AND METHODS

A. Formulation of Different treatments (Figure 1)

KSL-W was prepared into SLNs. The preparation of the lipids was based on a solvent free phase inversion method, which allowed for particle sizes ranging from 75-125 nm. 20.0 mg of emulsifying wax (Spectrum Chemical MFG. Corp) was mixed with 35.0 mg of surfactant Brij 78 (Spectrum Chemical MFG. Corp) and stirred at 350 rpms, 70 ^{O}C until the wax was fully melted. 7.8 ml of deionized water was added to the solution and stirring was increased to 1150 rpms. 200 µL of 50 mM SDS was combined with the lipid nanoparticle solution to give a final charge ~ -40 mV. In a separate flask, 2 mg of KSL-W was dissolved in 2 ml of deionized water. KSL-W, a positively charged cation, was added by drops to the SLN solution and stirred at 1150 rpms 70 ^{O}C for 25mins. The treatment solutions were returned to room temperature then filtered through a 0.2 µ filter and stored in 25mm vials at 15 ^{O}C for routine use or lypophilized (freeze dried) for long-term storage. Particle size and zeta potential were analyzed (90 Plus Particle Sizing Software Ver 4.03, Brookhaven, CT).

B. Cell Culturing Techniques (Figure 2)

Cell culturing technique

The culture method was recommend by Cell Applications, San Diego, CA. Cell culturing is essential for maintaining cells for assays. Aseptic technique was

always used to maintain clean culturing fields. Primary immortalized HaCaT [(cultured human keratinocytes (Cell Applications, San Diego, CA)] were thawed quickly in 37 ^{O}C water bath, centrifuged at 1000 rpms, 22 ^{O} C and freezing media was removed leaving the cell pellet. The cell pellet was resuspended in 10 ml of keratinocyte growth media and transferred to a T75 flask for storage at 37 ^{O}C, 5% CO_2. Growth media was replaced every 48 hours while the cells were being grown to confluence (Figure 2d). Once cells reached 60-70% confluence, the cells were split, passaged or frozen for preservation in cryotubes (Fisher Healthcare, Houston, TX). While splitting cell lines, the old media was removed and cells were washed twice in 10 ml of PBS 1x (Figure 2b). The cells were bathed in 5ml of 0.05% trypsin/EDTA (Sigma, St. Louis, MO) (2c) and incubated at 37 ^{O}C, 5% CO_2 for 5 minutes or until cells separated from flask floor. Cells were fully dislodged from the floor of the T75 flask with gentle wrapping on the table and reactions were neutralized with 5ml of keratinocyte growth media (Figure 2e and 2f). The solution was centrifuged at 1000 rpm for 5 minutes at 22^{o}C and the media removed. The pellet was resuspended with 2ml of new keratinocyte growth media. The cell count was completed by placement of 100 µL of cell suspension with100-µL trypan blue (1:1) (Sigma-Aldrich, St. Louis, MO). 10 µL of cell and trypan blue solution was analyzed by Countess ^{TM} Automated Cell Counter (Invitrogen, Grand Island, NY) (Figue 4c). The cell counter determined total cells, viable cells and percent of viable cells. Cells were then split or frozen down for later use. Cryopreservation was be completed by placement of cells in freezing media composed of 90% growth media

and10% DMSO (Sigma-Aldrich, Bellefonte, PA) with a final concentration of 2ml in a cryotube to be stored at -80 ^{O}C liquid nitrogen freezer.

C. ECIS Experimental Design (Figure 3)

ECIS 8W10E (Applied Biophysics, Troy, NY) plates (Figure 3c) were pretreated with 200 µL of 10 nM cysteine (Sigma-Aldrich, St Louis MO) (60.58 mg/ 50 ml ddH_2O) and were allowed sit at room temperature for 10-15 mins. They were rinsed with ddH_2O twice, drained and allowed to dry. ECIS wells were then inoculated with 1.25x10^5 cells/ cm^2 HaCaT cells (Lonza, Walkersville, MD) by using a stock solution of 3.125x10^5 cells/ mL in high glucose Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS) and 1% Streptomycin/Amphotericin (Lonza, Walkersville, MD) to a total volume of 400 mL per well. The cells were allowed to incubate at room temperature for 25-30 minutes to prevent clumping and allow for even distribution of cells on electrodes (Figure 3b). The ECIS 8W10E plate (Applied Biophysics, Troy, NY) was then reconnected to the ECIS platform (Figure 3d) and ECIS software (Applied Biophysics, Troy, NY) was set at multiple frequencies setting (16,000-24000 Hz). This allowed recording of low frequency barrier functions of cell-to-cell interactions and high frequency, recording the amount of substrate on the electrodes. At 12 hours, media was changed for 500 µL high glucose Dulbecco's Modified Eagle's Medium (Lonza, Walkersville, MD) with 1% Streptomycin/Amphotericin and no FBS. Wells were treated at 27.5 hrs with a

positive control of 2ng/ml transforming growth factor β1 (TGF- β1), with a negative control of 2μg/ml Somatostatin, and treatment groups of 200μg/ml KSL-W, 200μg/ml KSL-W+ SLN, or SLN. Cells were wounded at 28hrs with 6500 μA, 64,000 Hz for 20 seconds. Impedance was measured at treatment, prior to wounding and 1-hr, 5-hrs, and 24hrs post wounding.

D. Viable Cell Count (Figure 4)

$1x10^5$ cells were seeded in T-25 flasks with 3ml of high glucose Dulbecco's Modified Eagle's Medium (Lonza, Walkersville, MD), 1% Streptomycin/ Amphotericin, and 10% FBS. Media was incubated at 37 ^{O}C, 5% CO_2 and was changed at 48 hrs for FBS free media. Flasks were treated with a positive control of 2 ng/ml transforming growth factor β1 (TGF- β1), a negative control of 2 μg/ml of somatostatin and treatment groups of 200 μg/ml KSL-W, 200 μg/ml KSL-W+ SLN, or SLN (Figure 4a). Flasks were incubated for additional 12 hrs before cells were separated and analyzed. Digital photographs were acquired by 5x inverted light confocal microscopy pre-treatment and post-treatment for visual analysis (Figure 4b). Flasks were washed with 3 ml PBS 1x twice and separated with 2 ml 0.05 % trypsin/EDTA (Sigma, St. Louis, MO). Cells were removed, centrifuged at 1000 RPM for 5 minutes at 22 ^{O}C. Cells were resuspended in 1ml of HaCaT growth media and cell count acquired by Countess® Automated cell counter (Figure 4c).

Figure 1- Preparation of Treatment Groups

1a.

1b.

1c.

1d.

1e.

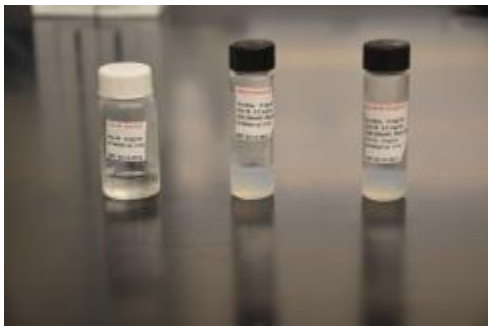
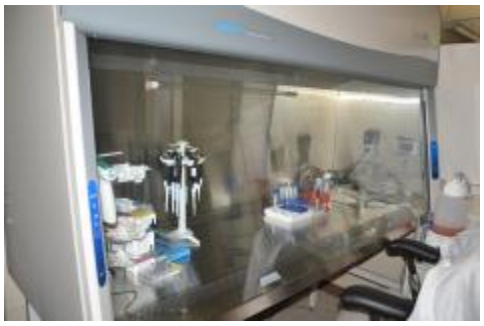


Figure 2- HaCat Cell Culturing Procedure

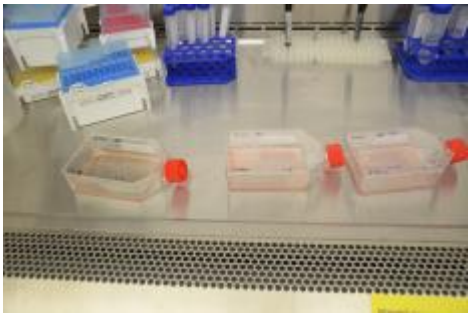
2a.



2b.

2c.

2d.



2e.

2f.

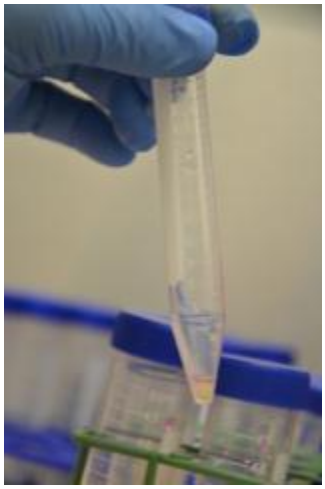
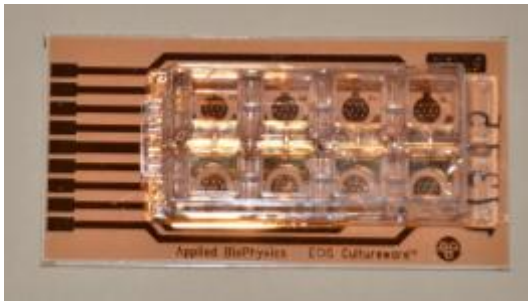


Figure 3- ECIS migration

3a.

3b.

3c.

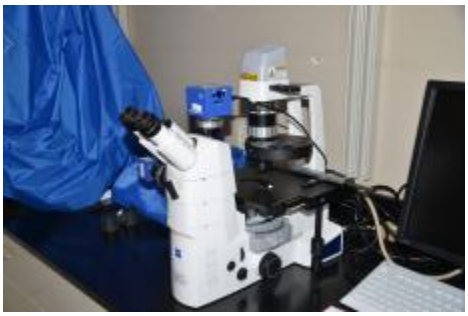


3d.

Figure 4- Cell Viability at 12 hrs

4a.

4b.



4c.

D. Statistical Management of Data

Mean and standard error were determined for each group and each time point of the ECIS migration. One-way ANOVA was used to evaluate the effects of the three different treatment groups (KSL-W, KSL-W + SLN and SLN) when compared to untreated cells, a positive migration control and a negative migration control measured by ECIS. Three different time points were examined to see the change in cell impedance (ohms) of the different treatment groups at 1 hr, 5 hrs and 24 hrs after treatment. Mean and standard deviation were used for each group while analyzing cell viability. One-way ANOVA was used to evaluate the effects of the three different treatment groups (KSL-W, KSL-W + SLN and SLN) when compared to untreated cells, positive and negative controls. All assays were analyzed using Minitab 16 and Statsplus. See Appendix A

IV. RESULTS

The statistical analysis was reviewed and approved by the clinical research administrator, Clinical Research Division, Joint Base San Antonio, Lackland AFB, TX. There was no significant difference found between the groups of KSL-W, KSL-W + SLN and SLN groups when compared to one another with Tukey Post-Hoc test. There was however differences found when KSL-W, KSL-W +SLN and SLN were compared to untreated cells, positive and negative controls with Tukey Post-Hoc test.

ECIS

Treatment with 200µg/ml KSL-W, 200µg/ml KSL-W +SLN and SLN at this formulation does not show to be beneficial to the migration of immortalized human keratinocytes when compared to untreated cells, 2 µg/ml somatostatin, and cells treated with 2 ng/ml TGF-β1. Significant differences were found between KSL-W, KSL-W + SLN and SLN when compared to control, positive control and negative control ($p<0.01$). There was however no significant difference when comparing the formulations KSL-W, KSL-W and KSL-W+ SLN ($p=0.523$). The different formulations (KSL-W, KSL-W+SLN and SLN) caused a significant reduction in the migration of HaCaT cells (Graph 1 and Table 1). The negative control (somatostatin) was not found to be significantly different when compared to untreated cells when compared with a Tukey post-hoc test. The negative control was removed and the results reconfirmed significant differences between treatment groups KSL-W, KSL-W + SLN and SLN when compared to untreated and positive control (Graph 2 and Table 2). This cessation in cell movement was also seen when examined under 5x confocal microscopy, which showed significantly less cell movement (see Appendix C).

A. ECIS: KSL-W and Controls (Appendix A, Figures 1-3A, 1-3B and 1-3C)

   Treatment of cells with KSL-W when compared to positive controls, negative controls and untreated cells were shown to have significant differences within the group with impedance when measured by ECIS at 5hrs ($p=0.049$) and 24hrs ($p =0.0002$) but not at one hour ($p=0.92$).

B. ECIS: SLN and Controls (Appendix A, Figures 4-6A, 4-6B and 4-6C). Treatment with cells with SLN when compared to positive control, negative controls and untreated cells were shown to have significant differences within the group with impedance when measured by ECIS at 5hrs ($p=0.003$) and 24hrs ($p =0.002$) but not at one hour ($p=0.24$).

C. ECIS: SLN + KSL-W and Controls (Appendix A, Figures 4-6A, 4-6B and 4-6C). Treatment with cells with SLN when compared to positive control, negative controls and untreated cells were shown to have significant differences within the group with impedance when measured by ECIS at 5hrs ($p=0.003$) and 24hrs ($p =0.002$) but not at one hour ($p=0.24$).

Cell Count and Photographic Analysis at 5x with confocal microscopy

A. Photographic analysis of Appendix C- Control pre-treatment and post-treatment: Pre-treatment and post-treatment of control cells showed no changes in cell morphology at the 12 hr time point. Cells showed integration with one another, flat and extended membranes with normal migratory patterns.

B. Photographic analysis of Appendix D- KSL-W pre-treatment and post-treatment: Pre-treatment and post-treatment of KSL-W treated cells showed no changes in cell morphology at the 12 hr time point. Cells

showed integration with one another, flat and extended membranes with normal migratory patterns.

C. Photographic analysis of Appendix E- KSL-W + SLN pre-treatment and post- treatment: Pre-treatment shows cell integration with one another, flat and extended membranes with normal migratory patterns. Post treatment of KSL-W + SLN shows dysplastic changes of cell membranes with round blebbing of cells and loss of extension seen during normal migratory patterns.

D. Photographic analysis of Appendix F- SLN pre-treatment and post-treatment: Pre-treatment shows cell integration with one another, flat and extended membranes with normal migratory patterns. Post treatment of KSL-W + SLN shows dysplastic changes of cell membranes with round blebbing of cells and loss of extension seen during normal migratory patterns.

E. Photographic analysis of Appendix G- Somatatostatin pre-treatment and post- treatment: Pre-treatment and post-treatment of cells with somatostatin showed no changes in cell morphology at the 12 hr time point. Cells showed integration with one another, flat and extended membranes with normal migratory patterns.

F. Photographic analysis of Appendix H- TNF-β1 pre-treatment and post-treatment: Pre-treatment and post-treatment of cells with TNF-β1 showed no changes in cell morphology at the 12 hr time point. Cells showed integration with one another, flat and extended membranes with normal migratory patterns.

Cell Counts 12hrs Post Treatment

Cell viability counts showed significant difference ($p>0.001$) with all treatment groups (Appendix B, Figure 7). Tukey post-hoc shows differences between SLN and KSL-W + SLN when compared to KSL-W and positive control, but no difference between untreated cells and negative control (Table 3).

Graph 1. Change in Ohms of Treatment Groups when Measured by ECIS

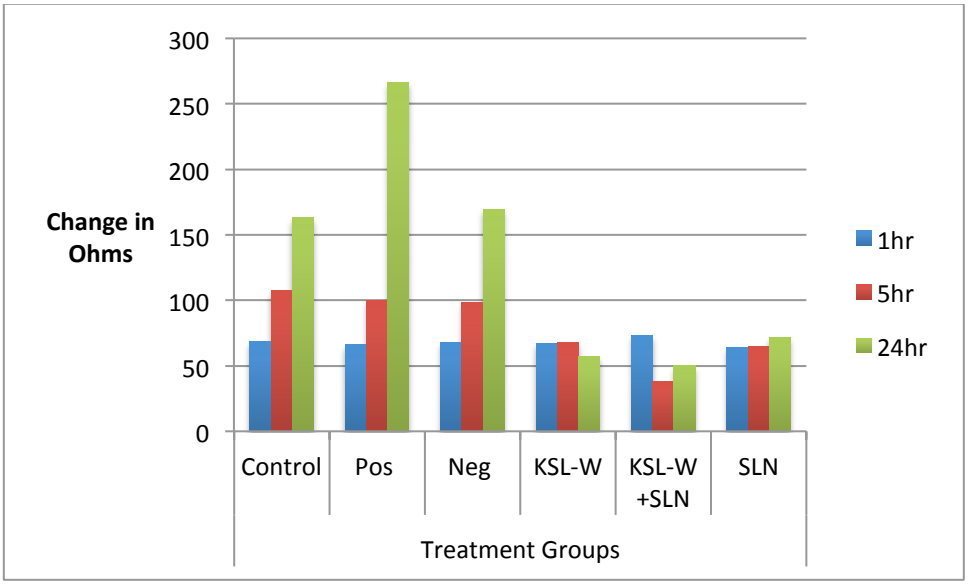


Table 1- Tukey Post-Hoc of Change in Ohms at 24hrs of All Groups

Treatment	Mean Impedance (Ohms) 24 Hours Post-Wounding	ANOVA
Control	163 ± 51.8	B
Pos	266.5 ± 87.1	A
Neg	169 ± 75.4	B
KSL-W + SLN	49.9 ± 19.5	C
SLN	71.4 ± 9.3	C
KSL-W alone	57.1 ± 35.5	C

Legend for Graph 1 and Table 1

Treatment groups:
Control: Untreated cells
Pos: Treated with TGF-β1
Neg: Treated with Somatostatin
KSL-W: Treated with KSL-W
KSL-W + SLN: Treated with solid lipid nanoparticle encapsulated KSL-W
SLN: Solid lipid nanoparticle only

Graph 2.- Change in Ohms of Groups Without Neg Control Measured by ECIS

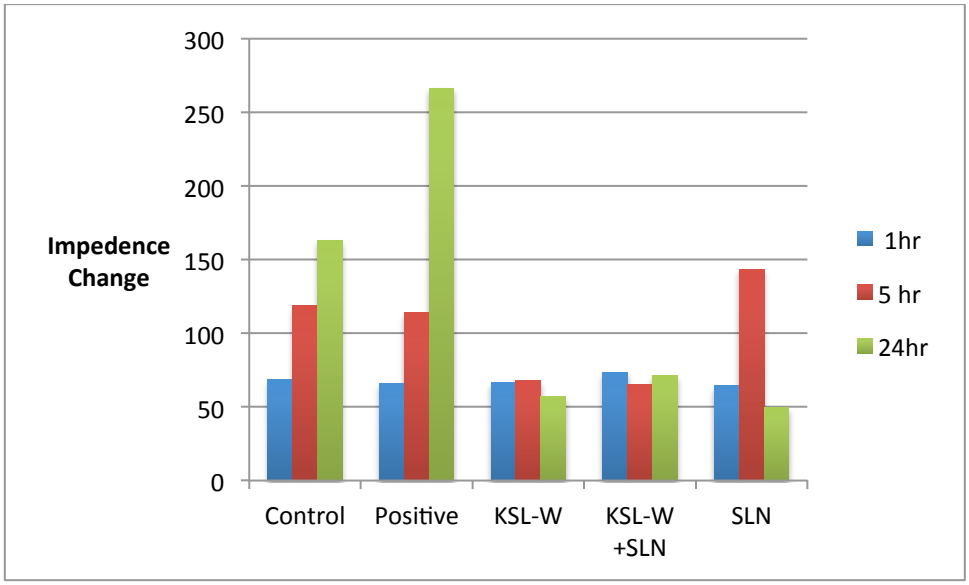


Table 2.- Tukey post-hoc of change in ohms of groups without neg control measured by ECIS

	Mean Impedance (Ohms) 24 Hours Post-Wounding	**ANOVA**
Control	163 ± 51.8	B
Pos	266.5 ± 87.1	A
KSL-W + SLN	49.9 ± 19.5	C
SLN	71.4 ± 9.3	C
KSL-W alone	57.1 ± 35.5	C

Legend for Graph 2 and Table 2

Treatment groups:
Control: Untreated cells
Pos: Treated with TGF-β1
KSL-W: Treated with KSL-W
KSL-W + SLN: Treated with solid lipid nanoparticle encapsulated KSL-W
SLN: Solid lipid nanoparticle only
Graph 3- Cell Viability at 12 hrs

Graph 3- Cell Viability at 12 hrs.

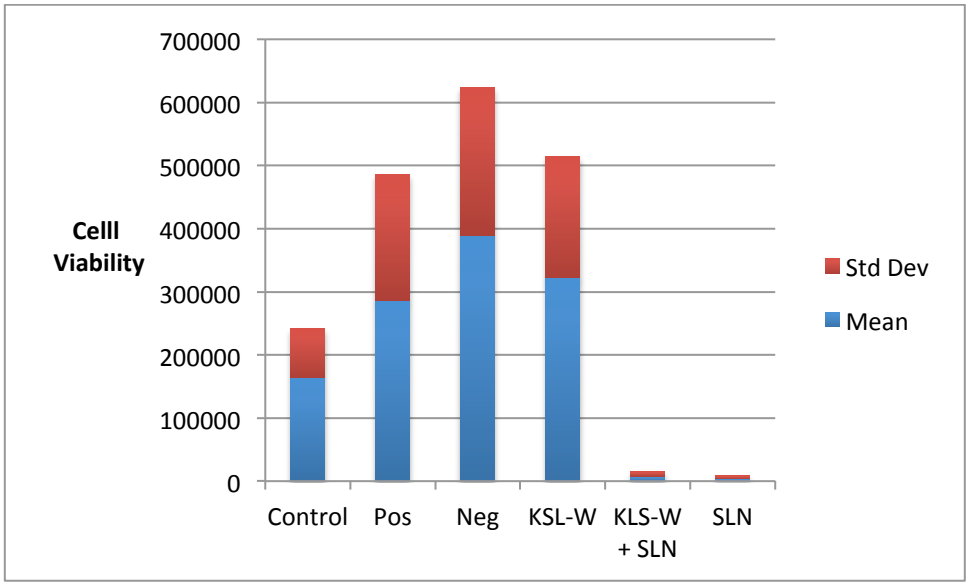


Table 3- Tukey Post-Hoc of Cell Viability at 12 hrs

Treatment	Cell Viability at 12hrs	ANOVA
Control	1.6 x10^5 ± 7.8x10^4	AB
Pos	2.6 x10^5 ± 2.0 x10^4	AB
Neg	3.5 x10^5 ± 2.3x10^4	A
KSL-W + SLN	8.3 x10^4 ± 7.5x10^3	B
SLN	8.3 x10^4 ± 5.1x10^3	B
KSL-W alone	2.8 x10^5 ± 1.9x10^4	A

Treatment groups:
Control: Untreated cells
Pos: Treated with TGF-β1
Neg: Treated with Somatostatin
KSL-W: Treated with KSL-W
KSL-W + SLN: Treated with solid lipid nanoparticle encapsulated KSL-W
SLN: Solid lipid nanoparticle only

V. DISCUSSION

The results of this study indicate a number of possibilities, which may explain why the KSL-W-, KSL-W + SLN and SLN-treated HaCaT cells decreased increase their impedance pattern when compared to the controls. ECIS studies have been used not only to compare cell migration but also cell viability in the presence of toxic elements (Xiao, 2003). One explanation of the cells' lower impedance could be due to the inability of the HaCaT cells to adhere to the polystyrene T-25 flasks and ECIS wells after treatment with SLNs. Recently, surfactants have been investigated for their ability to reduce bacterial adhesion of biofilms by up to 66% on surfactant treated polystyrene (Zeraik, 2010). The surfactant Brij 78, a major component in SLNs being used in this study, may have a similar effect with HaCaT cell adhesion to the polystyrene substrates of the T-25 flasks and ECIS wells. The inability to adhere would critically interfere with the cells ability to migrate over the ECIS sensor, preventing a raise in impedance. Keratinocytes also require adhesion to a substrate and without it they will initiate programed cell death (apoptosis) (Kikuchi, 1999). If surfactant Brij 78 prevents keratinocyte adhesion to the polystyrene flasks and ECIS wells, then they would initiate apoptopsis due to inability to attach, thus preventing an increase in impedance. This inability to attach may possibly explain the lack of change in impedance over ECIS sensors when compared to the controls and KSL-W treated cells. A recent study by Küchler and coworkers validated this theory. They completed a similar experiment to test the effects of SLN encapsulated morphine on a three-dimensional wound-healing model (Küchler, 2010). A SLN similar in

diameter size was used with surfactant Campritrol® for delivery of morphine (Küchler, 2010). They reported an accelerated rate of healing with SLN alone. Furthermore, they used a three-dimensional model of epidermis and dermis, which was composed of EpiDermFT^{TM} (MatTek, Ashland, MA) and contained both human fibroblasts and human keratinocytes. This model comparison shows two possible solutions that may indicate why there were drastically different results between experiments. The cell-to-cell interaction of this three-dimensional model allowed for paracrine interaction between cells of not only the keratinocyte level but also the fibroblast level. These paracrine interactions could have allowed better cell-cell communication allowing cell coordinated cell migration. Secondly, fibroblasts of the dermal layer may act as a buffer between the treatment groups and the polystyrene container. Their model treated the area directly over an incision, which contained fibroblasts at the base of the wound. The fibroblasts would likely interfere with SLNs ability to coat the polystyrene base thus providing better substructure for the superficial keratinocyte layer to migrate over.

The formulation of SLNs have also shown the risk of being cytotoxic based on the surfactant used. This was shown in an experiment Maupus *et al*. in which a variety of surfactants showed different levels of cytotoxicity to HaCaT cells (Maupus, 2011). Though they did not examine Brij 78 the surfactant used in this study, it does present and inherent potential to have similar cytotoxic properties to the surfactants examined. Surfactants are readily interchangeable with other surfactants within formulation and present similar possibilities for drug delivery formulation changes if

the surfactant is noted for being responsible for decreased cell migration and cytotoxicity (Mottaleb, 2011).

Somatostatin was used for the negative control based on the Vockel, 2011 study that reported reduced migration and cell counts due to delayed lamellipodia formation effecting cytoskeleton development. This is conflicting data when compared to a prior study completed Hegerstrand, 1989 and evidence found in this in this study. The Vockel study was completed with a scratch assay which implements linear analysis with photographic evaluation of cells and gene confirmation of regulation. The ECIS assay, though automated provides limited information on cell morphology change, and the migration pattern is in a circular pattern, which could provide for the difference in results.

Keratinocyte migration alters cell phenotype from a stationary basal cell to migratory phenotype, which is defined when the cells flatten and elongate (O'Toole, 2001). This is done by extension of lamelipodia and redistribution of keratin filaments (O'Toole, 2001). Apoptosis of cells is defined as automated cell death. When this occurs with keratinocytes, cultures show blebbing of cells and freeing of epithelial sheets (Chen, 2002). This is noted in the photographic analysis in Figure 7 and 8, which used a combination of SLNs. However, it is not noted in the control group or the KSL-W treatment groups indicating incompatibility of the SLNs groups.

VI. CONCLUSION

The formulation of SLN encapsulated KSL-W appears to have an effect on the migration of keratinocytes when analyzed by ECIS. Somatostatin used at 2 μg/ml does not slow migration significantly when compared to untreated cells. The drug delivery agents of KSL-W needs further studies to evaluate other preparations that would aid in its ability to remain viable over longer periods of time. Follow-up experiments should include MTT colorimetric assay to measure enzymatic activity and cytotoxicity of HaCaT cells with the different treatment groups used. Further studies shouid include, terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assays to evaluate apoptosis of cells treated with different groups. KSL-W + SLN should also be investigated on a three-dimensional skin model to evaluate the effect SLN when polypropylene surface is removed.

Appendix A- Raw ECIS Data and One Way ANOVA by Group

1A. Normalized Change in Ohms KSL-W T-0 to T -1hr

	Control	Pos	Neg	KSL-W
Normalized	69.2	57	51.5	61
	76.2	76.5	64.9	75.1
	86.9	81.2	82.3	73.3
	78	75.5	69.9	65.4
	48.5	63.3	62.5	73.1
	60.9	59.5	65.7	53.5
	Control	**Pos**	**Neg**	**KSL-W**
Mean	69.95	68.83	66.13	66.90
SEM	5.58	4.14	4.10	3.48

Analysis of Variance (One-Way)

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	419.7	69.95	186.747
Pos	6	413.	68.83333	102.78267
Neg	6	396.8	66.13333	100.91867
KSL-W	6	401.4	66.9	72.652

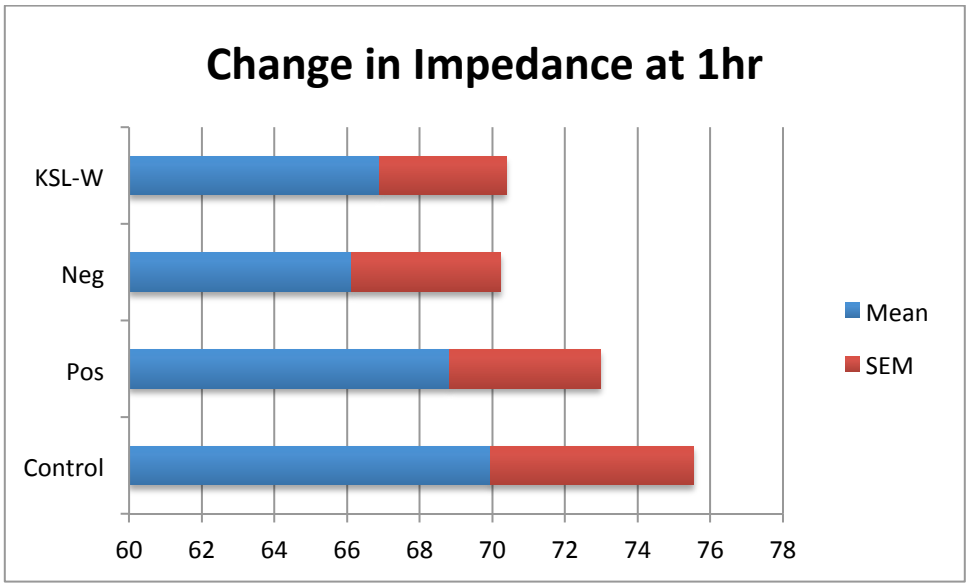
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	55.09792	3	18.36597	0.15863	0.92287	4.1134
Within Groups	2,315.50167	20	115.77508			

Total	2,370.59958	23

1B. One Way ANOVA of KSL-W T-0 to T-1hrs

1C. Change in Impedance of KSL-W at 1hr



2A. Normalized Change in Ohms KSL-W T-0 to T- 5hrs

	Control	Pos	Neg	KSL-W
Normalized	108.1	74.4	72.9	51.8
	110.6	123.2	96.2	70.7
	152.6	114.7	27.5	72.
	118.6	130.4	130.4	75.6
	69.9	90.8	84.7	80.2
	88.9	95.4	110.8	58.3
	Control	**Pos**	**Neg**	**KSL-W**
Mean	108.12	104.82	87.08	68.10
SEM	11.44	8.76	14.47	4.42

2B. One Way ANOVA of KSL-W T-0 to T-5hrs

Analysis of Variance (One-Way)

Summary

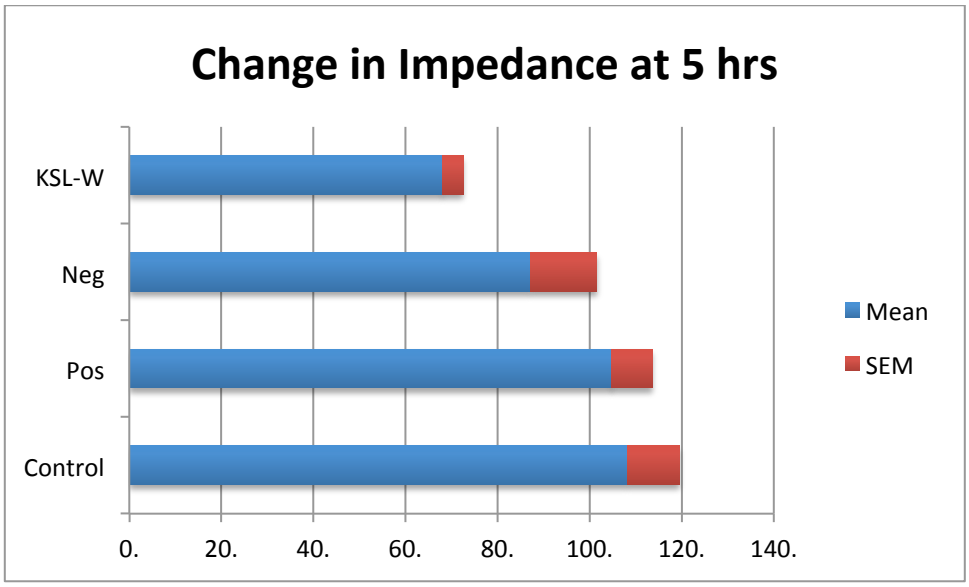
Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	648.7	108.11667	784.92567
Pos	6	628.9	104.81667	460.08967
Neg	6	522.5	87.08333	1,255.78967
KSL-W	6	408.6	68.1	117.272

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	6,116.36458	3	2,038.78819	3.11494	0.04923	4.1134
Within Groups	13,090.385	20	654.51925			

Total	19,206.74958	23

2C. Change in Impedance of KSL-W at 5 hrs



3A. Normalized Change in Ohms KSL-W T-0 to T- 24hrs

	Control	Pos	Neg	KSL-W
Normalized	140.57	74.66	51.23	0.27
	114.58	181.84	87.83	34.8
	263.84	320.69	200.35	71.74
	150.98	340.13	252.33	76.83
	82	281.5	140.8	101.5
	131.9	340	241.3	57.7
	Control	**Pos**	**Neg**	**KSL-W**
Mean	147.31	256.47	162.31	57.14
SEM	25.31	43.72	33.73	14.49

3B. One Way ANOVA of KSL-W T-0 to T-24hrs

Analysis of Variance (One-Way)

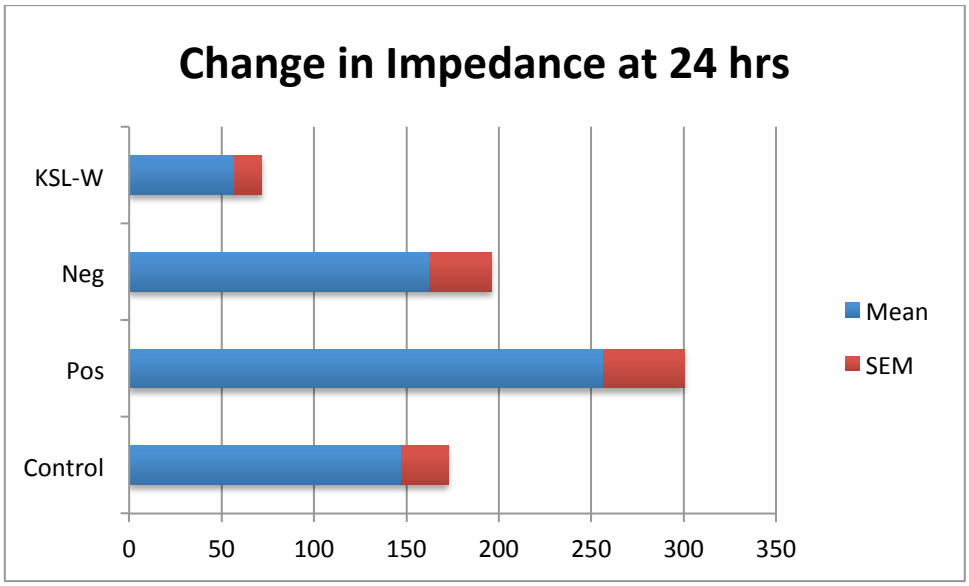
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	883.87	147.31167	3,842.4509
Pos	6	1,538.82	256.47	11,470.29576
Neg	6	973.84	162.30667	6,827.75579
KSL-W	6	342.84	57.14	1,260.45036

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	119,895.79688	3	39,965.26563	6.83139	0.00237	4.1134
Within Groups	117,004.76402	20	5,850.2382			
Total	236,900.5609	23				

3C. Change in Impedance of KSL-W at 24 hrs



4A. Normalized Change in Ohms SLN T-0 to T- 1hrs

	Control	Pos	Neg	SLN
Normalized	80.6	68.6	73.3	72.8
	77.8	70.2	72.1	61.1
	71.6	65.8	64	61
	70	70	70	70
	64.21	64.3	71.99	63.53
	61.57	53.42	61.76	57.37
	Control	**Pos**	**Neg**	**SLN**
Mean	70.96	65.39	68.86	64.3
SEM	3.03	2.58	1.96	2.41

4B. One Way ANOVA of SLN T-0 to T-1hrs

Analysis of Variance (One-Way)

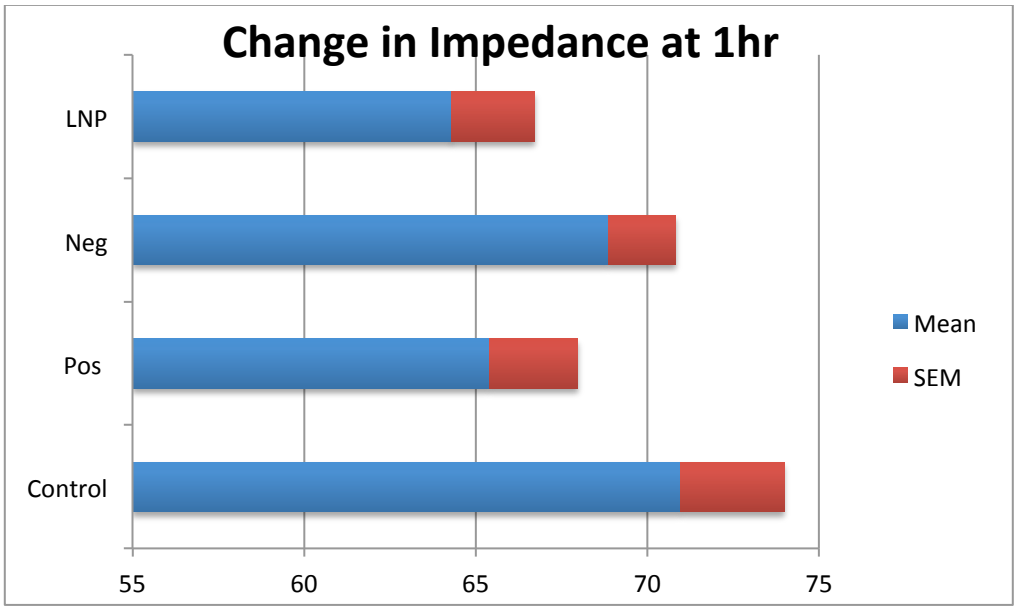
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	425.78	70.96333	54.95619
Pos	6	392.32	65.38667	39.86587
Neg	6	413.15	68.85833	23.06746
SLN	6	385.8	64.3	34.89756

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	170.91295	3	56.97098	1.49151	0.24723	4.1134
Within Groups	763.93535	20	38.19677			
Total	934.8483	23				

4C. Change in Impedance of SLN at 1hr



5A. Normalized Change in Ohms SLN T-0 to T- 5hrs

	Control	Pos	Neg	LNP
Normalized	112.8	94	88.3	68.6
	119.5	89.2	98.3	71
	130.4	149.7	99.4	63
	79.7	110.5	166.9	69.9
	125.62	88.99	106.19	65.03
	103.08	86.01	100.8	52.81
	Control	**Pos**	**Neg**	**LNP**
Mean	111.85	103.07	109.98	65.06
SEM	7.54	9.99	11.63	2.74

5B. One Way ANOVA of SLN T-0 to T-5hrs

Analysis of Variance (One-Way)

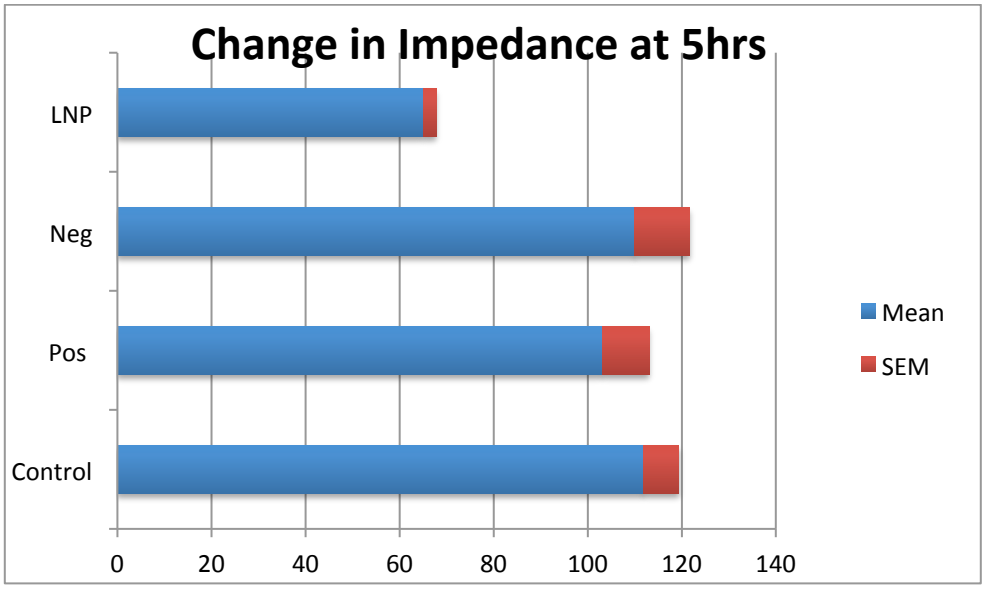
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	671.1	111.85	340.73516
Pos	6	618.4	103.06667	598.69871
Neg	6	659.89	109.98167	811.38082
SLN	6	390.34	65.05667	45.10955

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	8,671.62991	3	2,890.5433	6.43801	0.00315	4.1134
Within Groups	8,979.62115	20	448.98106			
Total	17,651.25106	23				

5C. Change in Impedance of SLN at 5 hrs



6A. Normalized Change in Ohms SLN T-0 to T- 24hrs

	Control	Pos	Neg	SLN
Normalized	115.48	168.53	92.59	69.4
	134.96	124.35	109.7	66.12
	185.41	379.95	239.59	77.93
	83.42	370.06	319.3	84.08
	209.11	279.4	171.65	73.32
	160.98	230.1	162.04	57.64
	Control	**Pos**	**Neg**	**SLN**
Mean	148.22	258.73	182.47	71.42
SEM	18.88	42.63	34.58	3.78

6B. One Way ANOVA of SLN T-0 to T-24hrs

Analysis of Variance (One-Way)

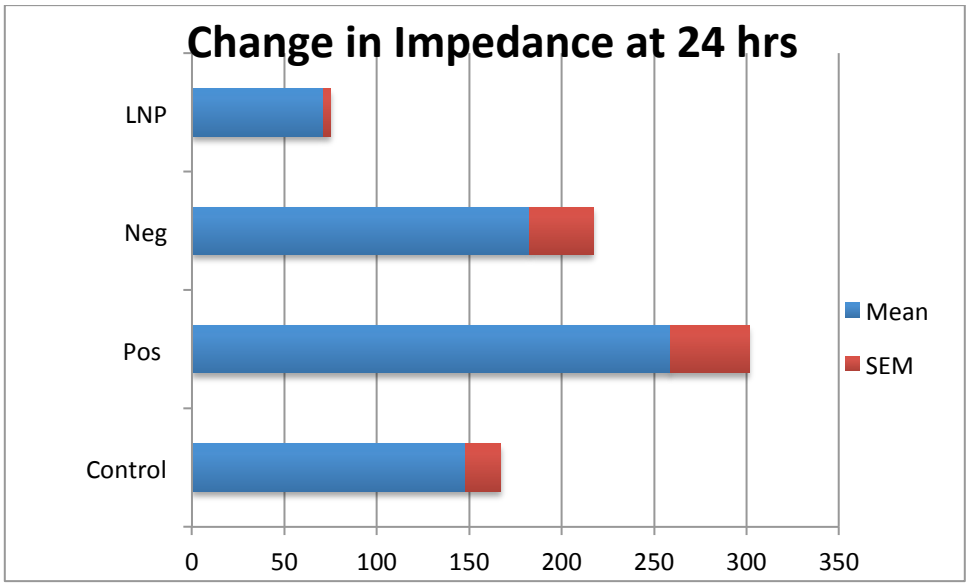
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	889.36	148.22667	2,140.0561 5
Pos	6	1,552.3 9	258.73167	10,905.921 5
Neg	6	1,094.8 7	182.47833	7,178.6975
SLN	6	428.49	71.415	85.66487

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	108,782.5984 5	3	36,260.8661 5	7.14136	0.0019 1	4.11 34
Within Groups	101,551.7000 5	20	5,077.585			
Total	210,334.2985	23				

6C. Change in Impedance of SLN at 24 hrs



7A. Normalized Change in Ohms KSL-W + SLN T-0 to T- 1hrs

	Control	Pos	Neg	KSL-W +SLN
Normalized	63.5	67.4	64.9	88.9
	66.7	65	76.4	78.1
	67.4	57.8	66.8	99.3
	64	63	64.5	80
	68.1	67.2	74.5	53.8
	64.2	64.1	68.7	40.7
	Control	**Pos**	**Neg**	**KSL-W +SLN**
Mean	65.65	64.08	69.30	73.47
SEM	0.81	1.44	2.05	9.00

7B. One Way ANOVA of KSL-W + SLN T-0 to T-1hrs

Analysis of Variance (One-Way)

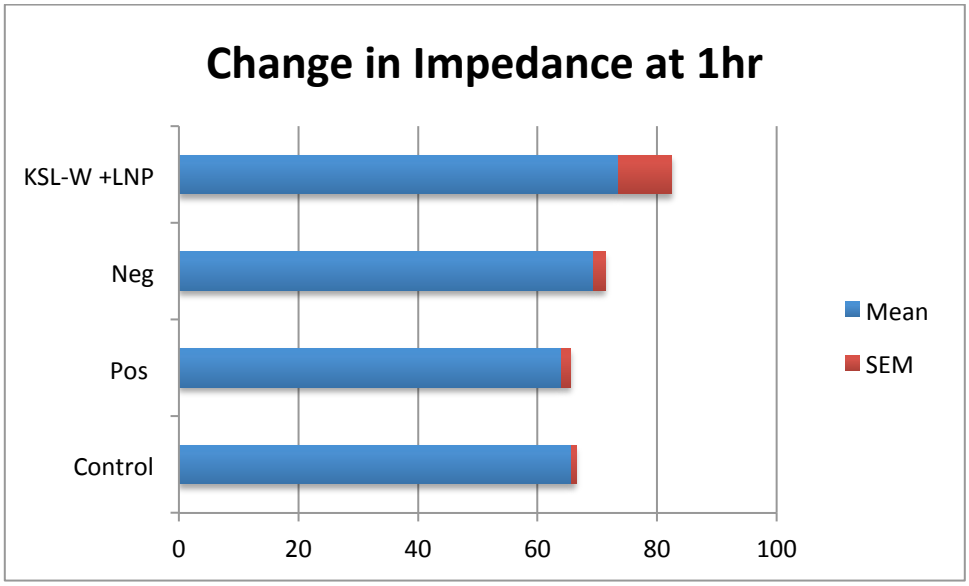
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	393.9	65.65	3.923
Pos	6	384.5	64.08333	12.44167
Neg	6	415.8	69.3	25.292
KSL-W +SLN	6	440.8	73.46667	486.02667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	314.24833	3	104.74944	0.79403	0.51153	4.1134
Within Groups	2,638.41667	20	131.92083			
Total	2,952.665	23				

7C. Change in Impedance of KSL-W + SLN at 1hr


Change in Impedance at 1hr
KSL-W +LNP
Neg
Pos
Control
0
20
40
60
80
100
Mean
SEM

8A. Normalized Change in Ohms KSL-W + SLNT-0 to T- 5hrs

	Control	Pos	Neg	KSL-W +SLN
Normalized	88.7	92.2	65.6	43.5
	87.2	77.1	117.2	18.8
	119.66	102.43	85.7	14.96
	109.02	91.03	104.42	64.18
	114.06	81.8	126.49	48.59
	92.1	106.1	95.6	36.1
	Control	**Pos**	**Neg**	**KSL-W +SLN**
Mean	101.79	91.78	99.17	37.69
SEM	5.77	4.60	8.98	7.59

8B. One Way ANOVA of KSL-W + LNP T-0 to T-5hrs

Analysis of Variance (One-Way)

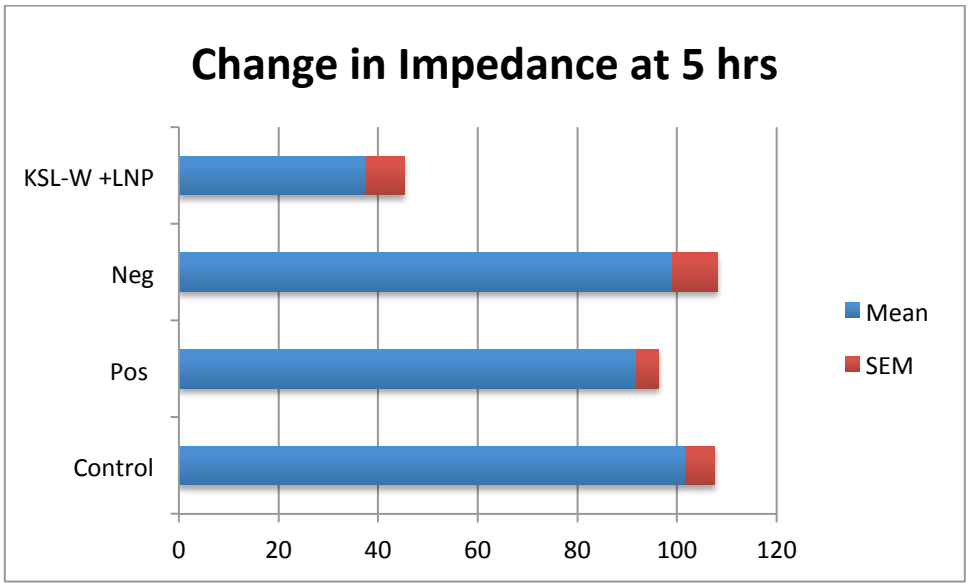
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	610.74	101.79	200.055
Pos	6	550.66	91.77667	126.86531
Neg	6	595.01	99.16833	484.0313
KSL-W +SLN	6	226.13	37.68833	346.05986

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	16,464.20788	3	5,488.06929	18.97326	0.	4.1134
Within Groups	5,785.0573	20	289.25287			
Total	22,249.26518	23				

8C. Change in Impedance of KSL-W + SLN at 5hr



9A. Normalized Change in Ohms KSL-W + SLN T-0 to T- 24hrs

	Control	Pos	Neg	KSL-W +SLN
Normalized	164.24	239.535	74.04	48.15
	130.6	263.3	177.2	34
	219.6	327.35	154.63	27.18
	231.5	312.3	193.9	82.9
	211.6	243.67	219.45	55.88
	203.6	321	160.8	51.5
	Control	**Pos**	**Neg**	**KSL-W +SLN**
Mean	193.52	284.53	163.34	49.94
SEM	15.67	16.41	20.27	7.96

9B. One Way ANOVA of KSL-W + LNP T-0 to T-24hrs

Analysis of Variance (One-Way)

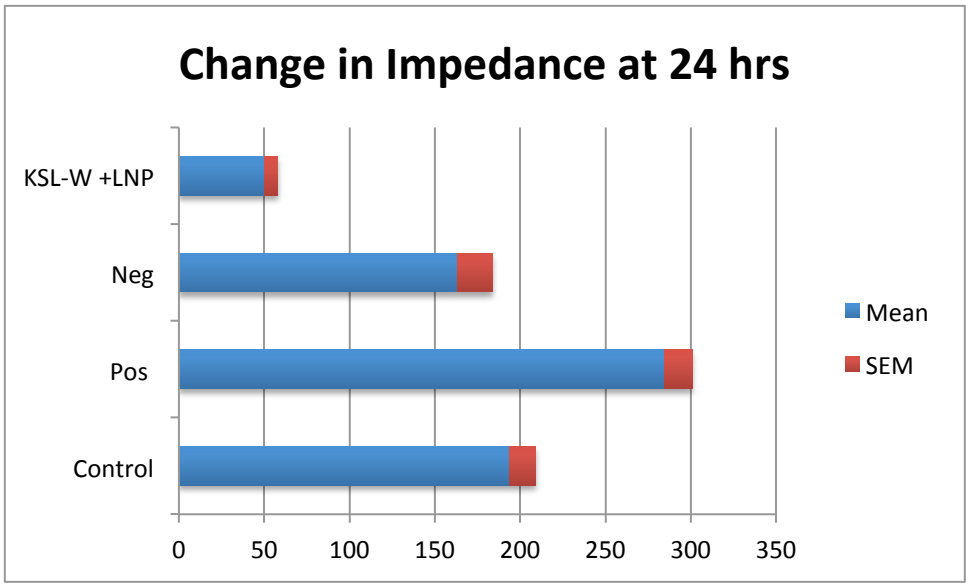
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	1,161.14	193.52333	1,473.47687
Pos	6	1,707.155	284.52583	1,615.91772
Neg	6	980.02	163.33667	2,466.23019
KSL-W +SLN	6	299.61	49.935	379.87679

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	168,584.86579	3	56,194.95526	37.8704	0.	4.1134
Within Groups	29,677.50784	20	1,483.87539			
Total	198,262.37362	23				

9C. Change in Impedance of KSL-W + SLN at 24 hrs



10. One Way Anova of Controls

Analysis of Variance (One-Way)

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Con	18	2,934.37	163.02056	2,685.65919
Pos	18	4,798.365	266.57583	7,227.99075
Neg	18	3,048.73	169.37389	4,936.00813

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	121,273.67042	2	60,636.83521	12.25015	0.00005	4.22805
Within Groups	252,444.18733	51	4,949.88603			
Total	373,717.85776	53				

APPENDIX B- Raw Cell Count Data and One Way ANOVA by Group

1. Cell Count of Control Group

Controls	Total Cells	Live	Viability
C1	200000	160000	78%
C2	190000	120000	61%
C3	180000	130000	74%
C4	80000	60000	80%
C5	320000	260000	81%
C6	320000	250000	77%
Mean	218000	164000	75.2%
Std Dev	92032.6	78145.2	

2. Cell Count of KSL-W Group

KSL-W	Total Cells	Live	Viability
K1	120000	86000	65%
K2	360000	270000	74%
K3	260000	200000	75%
K4	170000	120000	73%
K5	640000	580000	91%
K6	500000	440000	88%
Mean	386000	322000	83.4%
Std Dev	199841.6	192506.2	

3. Cell Count of KSL-W + SLNs Group

KSL-W + LNP	Total Cells	Live	Viability
KL1	40000	10000	13%
KL2	20000	0	0
KL3	70000	20000	31%
KL4	40000	0	0
KL5	30000	10000	20%
KL6	20000	10000	28%
Mean	36000	8000	22.2%
Std Dev	18619.0	7527.7	

4. Cell Count of SLNs Group

SLNs	Total Cells	Live	Viability
L1	10000	0	0
L2	10000	0	0
L3	40000	10000	28%
L4	10000	0	0
L5	20000	10000	33%
L6	10000	0	0
Mean	18000	4000	22.2%
Std Dev	12110.6	5164.0	

5. Cell Count of TNF-β1

TNF- β1	Total Cells	Live	Viability
T1	130000	120000	95%
T2	140000	140000	97%
T3	130000	100000	71%
T4	180000	160000	76%
T5	610000	530000	88%
T6	560000	500000	90%
Mean	576000	538000	93.4%

Std Dev	542758.4	537491.1	

6. Cell Count of Somatostatin

Somatostatin	Total Cells	Live	Viability
S1	170000	150000	91%
S2	320000	300000	95%
S3	300000	300000	95%
S4	180000	130000	74%
S5	600000	460000	76%
S6	850000	760000	95%
Mean	156600	147000	93.9%
Std Dev	1273427.1	1265096.8	

7. One- Way ANOVA of Cell Viability Count

Analysis of Variance (One-Way)

Summary

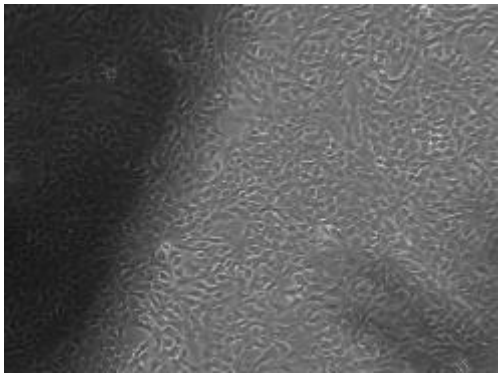
Groups	*Sample size*	*Sum*	*Mean*	*Variance*
Control	6	980,000.	163,333.33333	6,106,666,666.6667
Pos	6	1,550,000.	258,333.33333	4.00167E+10
Neg	6	2,100,000.	350,000.	5.472E+10
KSL-W	6	1,696,000.	282,666.66667	3.70587E+10
KLS-W +SLN	6	50,000.	8,333.33333	56,666,666.66667
SLN	6	20,000.	3,333.33333	26,666,666.66667

ANOVA

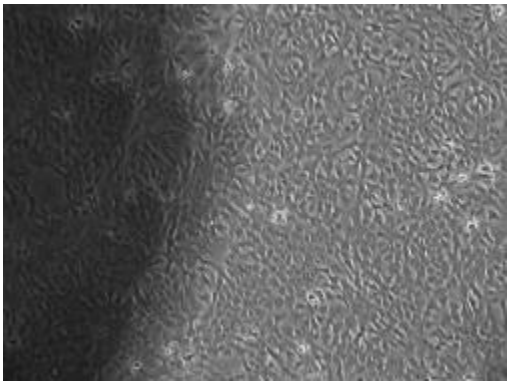
Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	6.39013E+11	5	1.27803E+11	5.55723	0.00097	3.1878
Within Groups	6.89927E+11	30	2.29976E+10			
Total	1.32894E+12	35				

Appendix C. Photographs at 5x Control Pre (A) and Post (B) treatment

1A.



1B.



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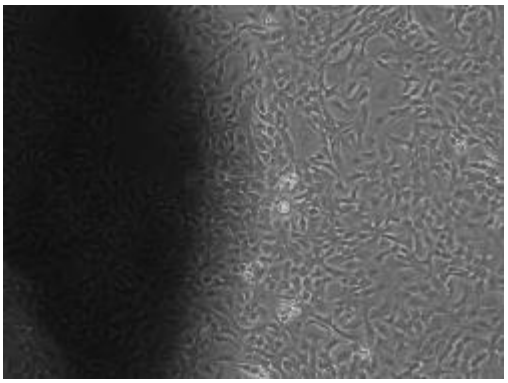
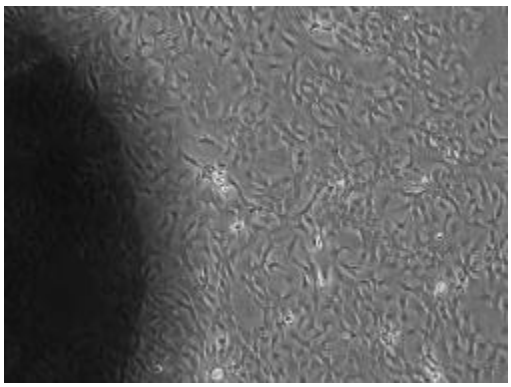
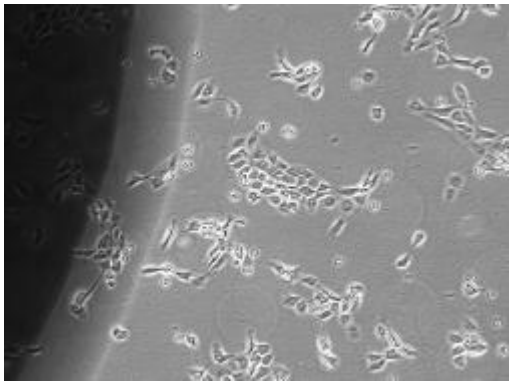


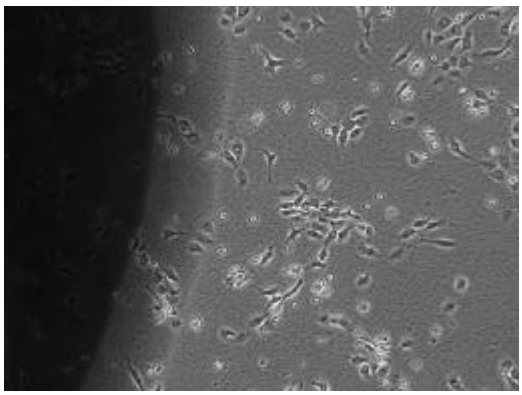
2B.



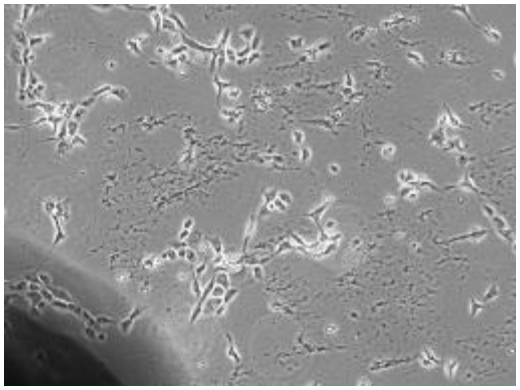
3A.



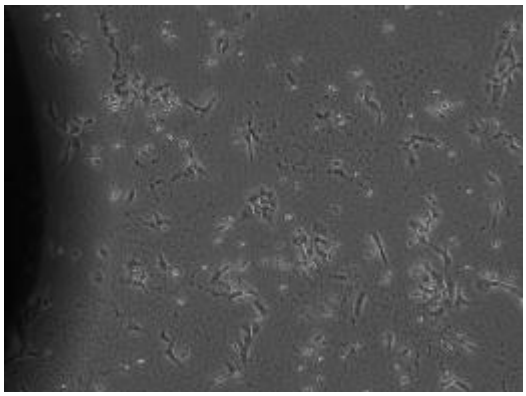
3B.



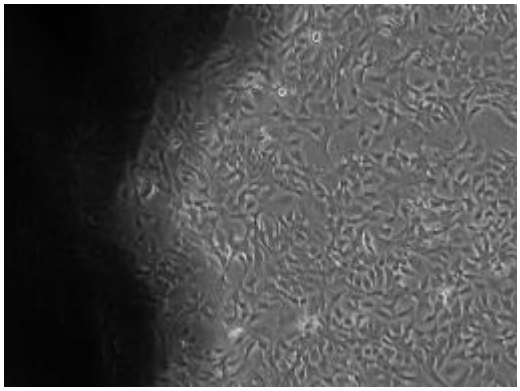
4B.



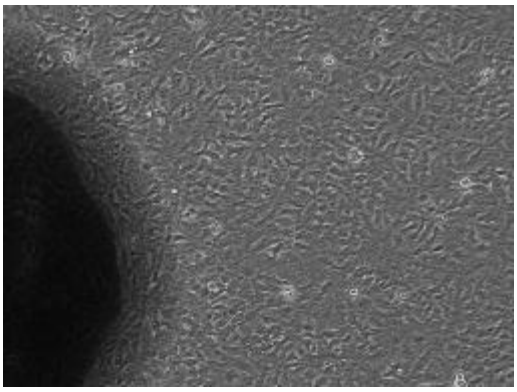
4C.



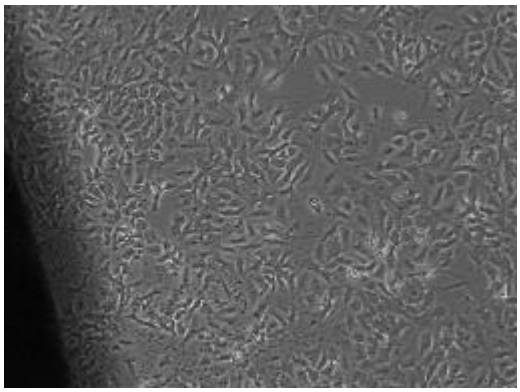
5A.



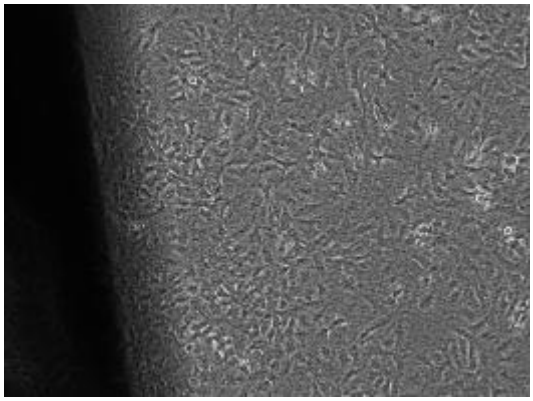
5B.



6A.

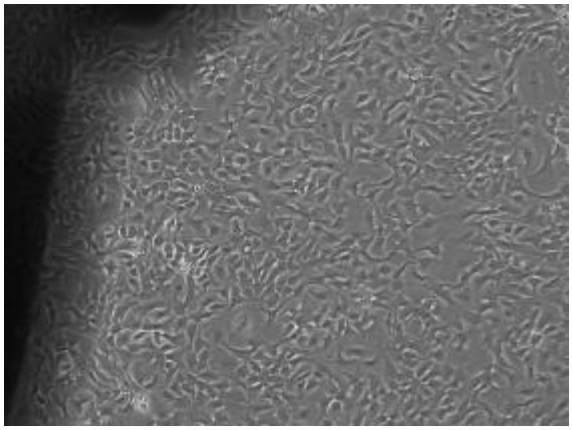


6B.

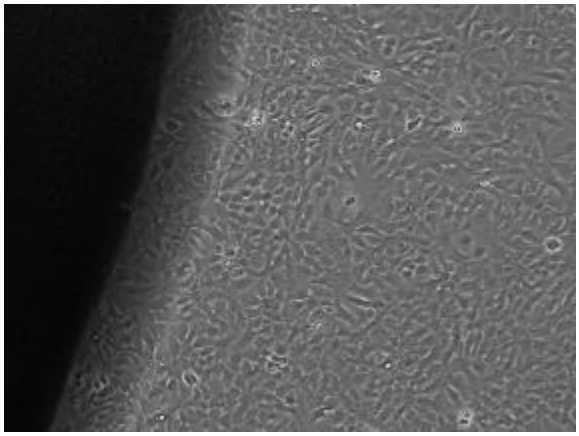


Appendix D. Photographs at 5x of KSL-W Pre (A) and Post (B) Treatment

1A.



1B.



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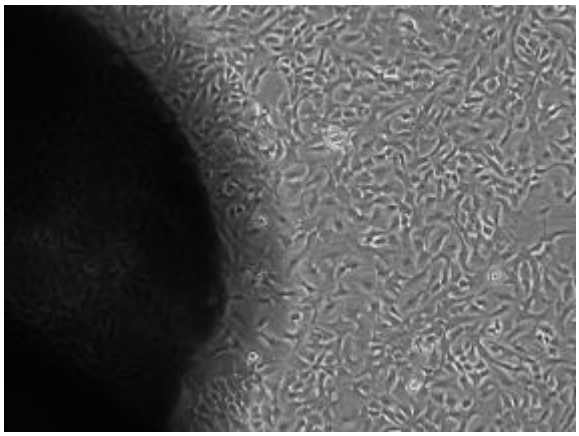
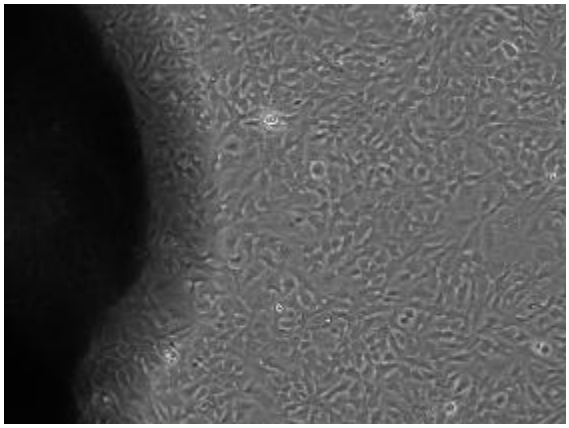
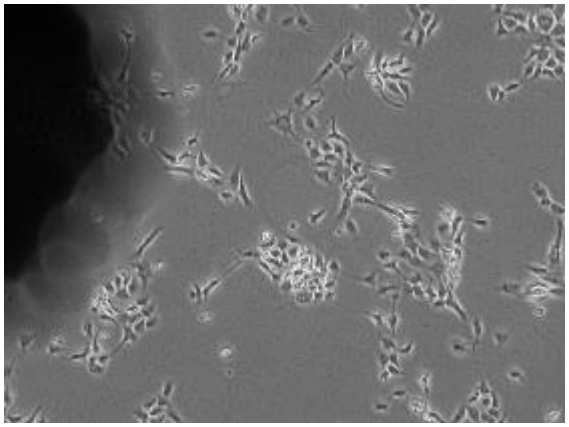


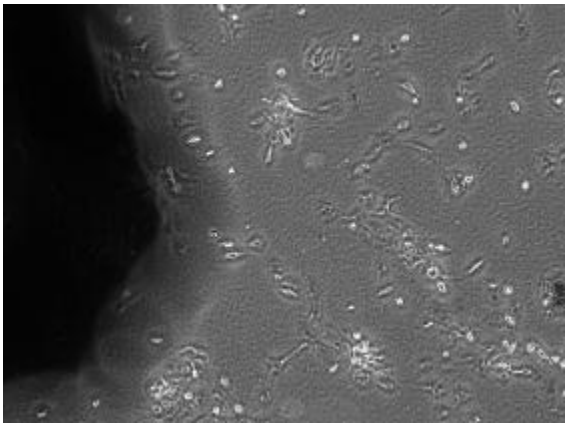
2B.



3A.



3B.



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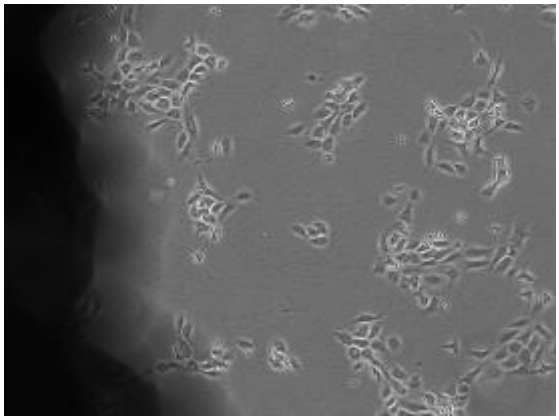
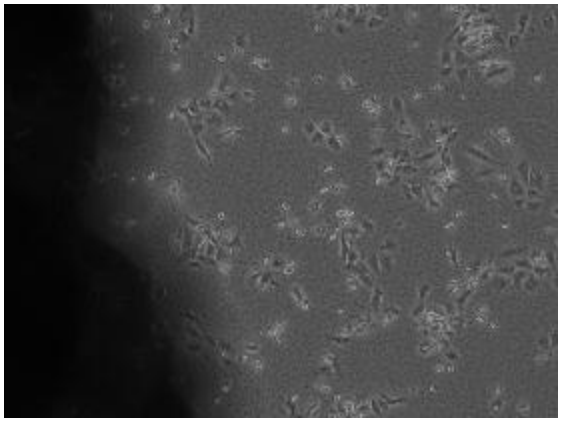
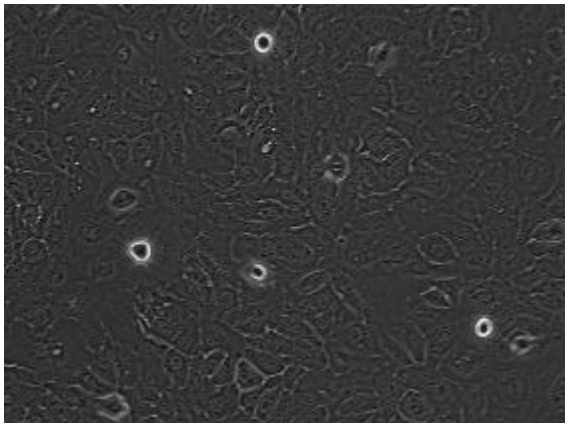


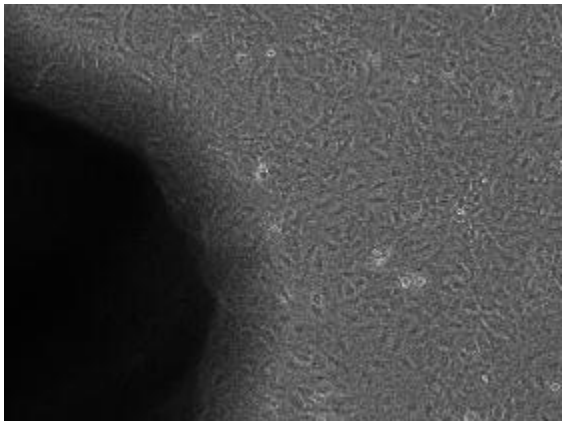
4B.



5A.

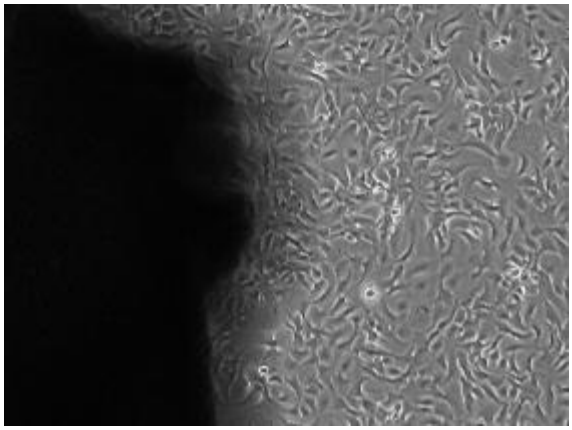


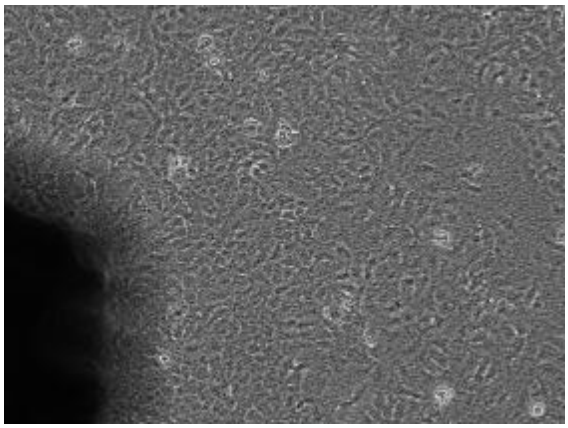
5B.



6A.

6B.

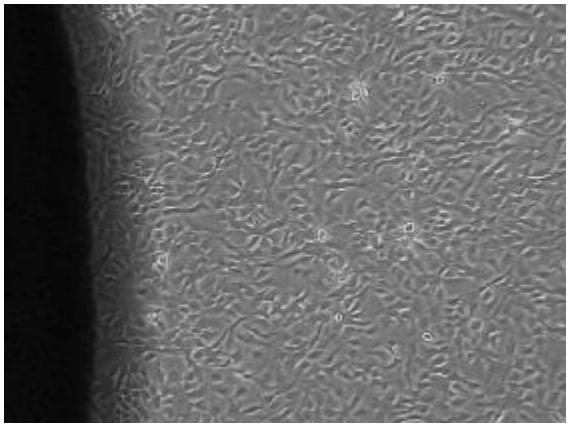




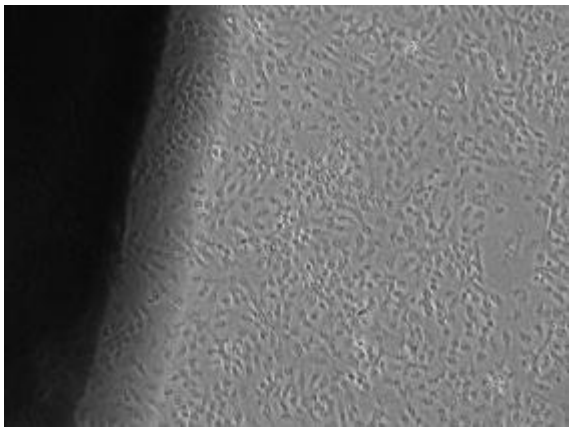
Appendix E. Photographs at 5x of KSL-W + SLN Pre (A) and Post (B)

Treatment

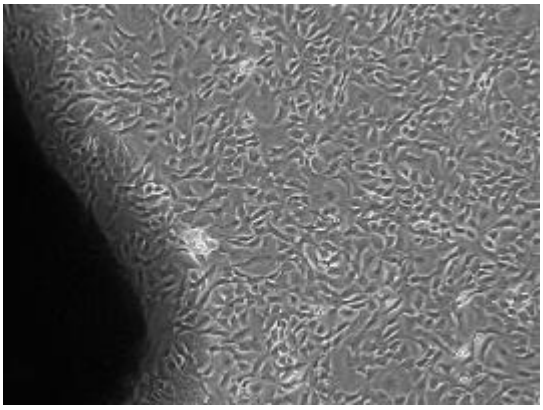
1A.



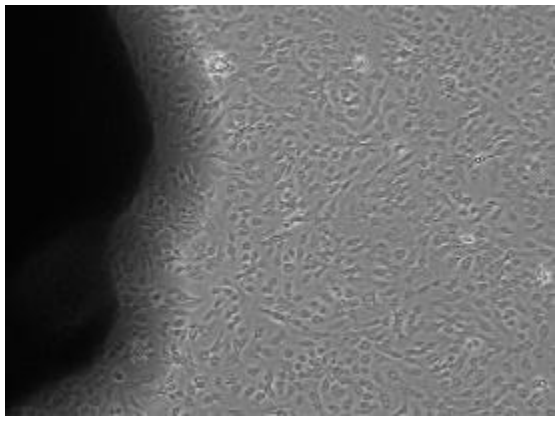
1B.



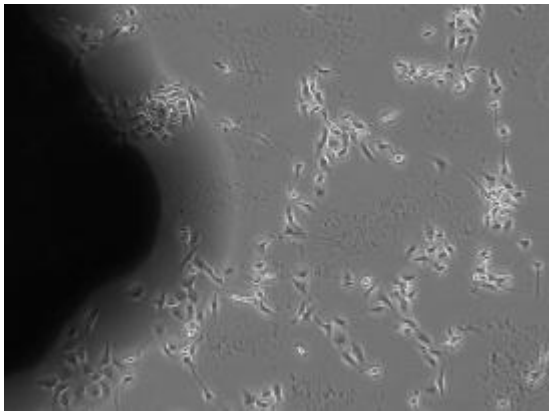
2A.



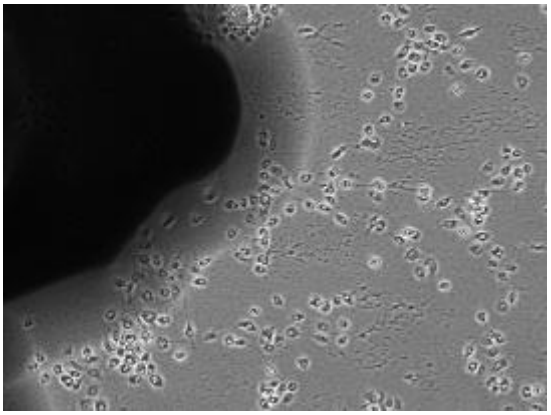
2B.



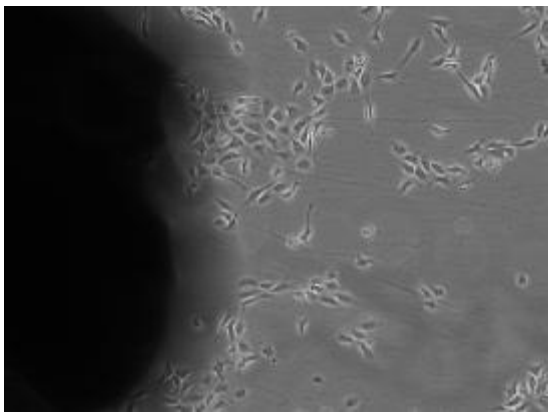
3A.



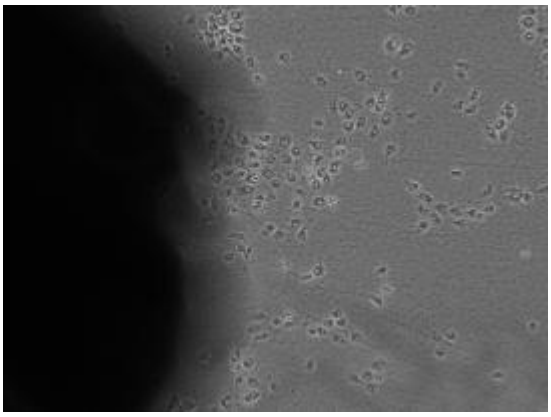
3B.



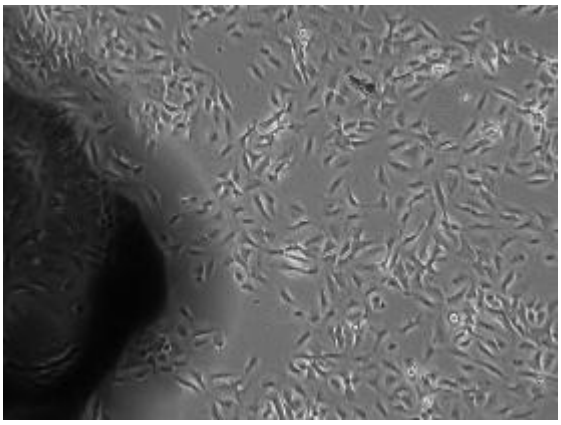
4A.



4B.



5A.



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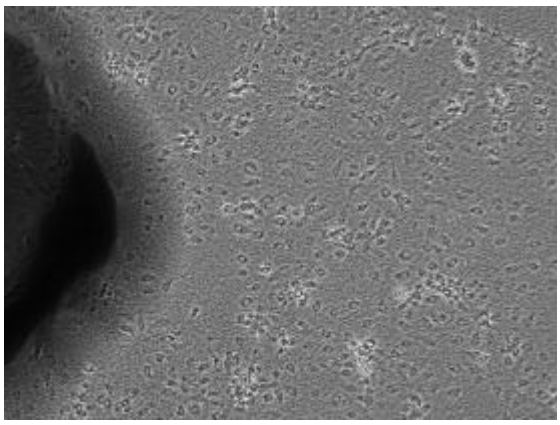
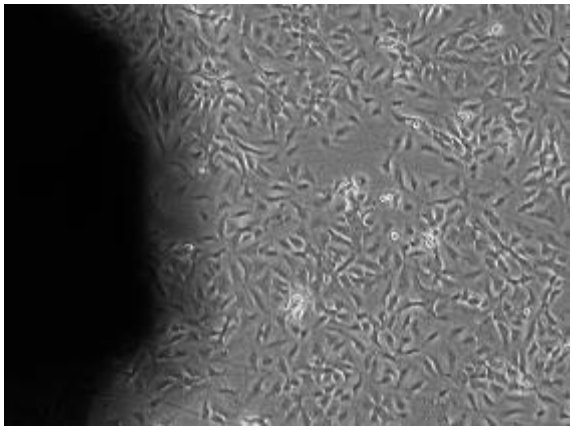
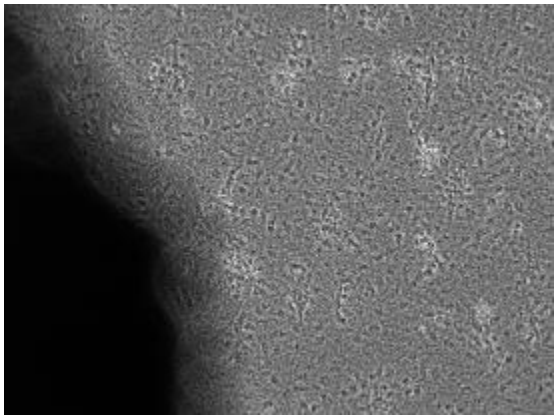


6A.

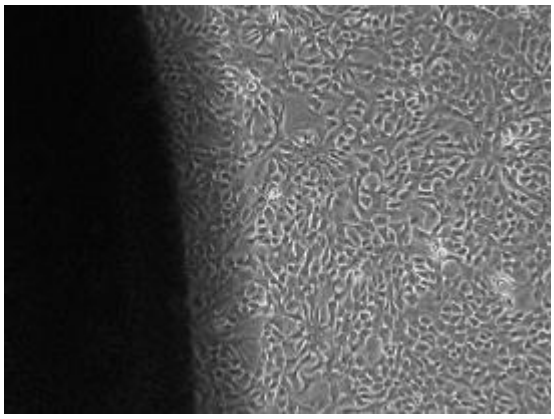
6B.



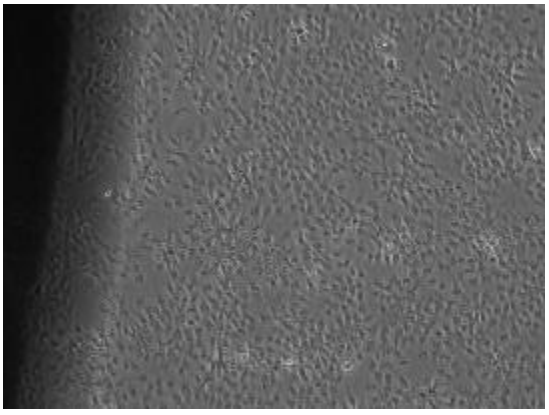


Appendix F. Photographs at 5x of SLN Pre (A) and Post (B) Treatment

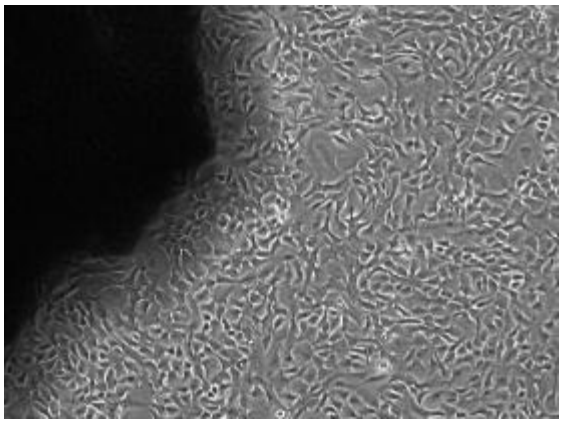
1A.



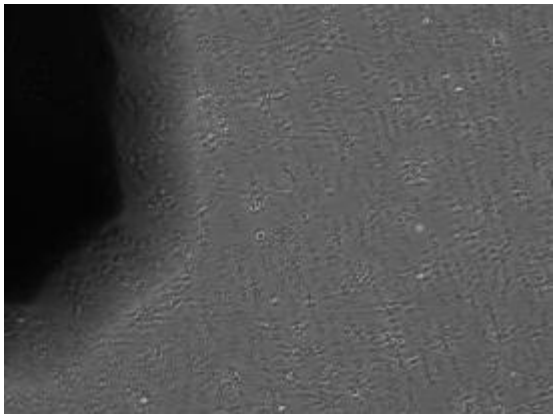
1B.



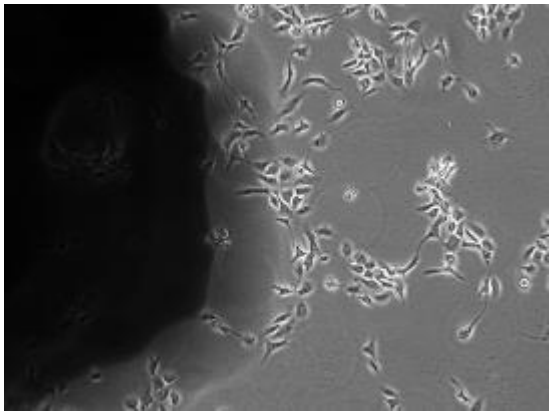
2A.



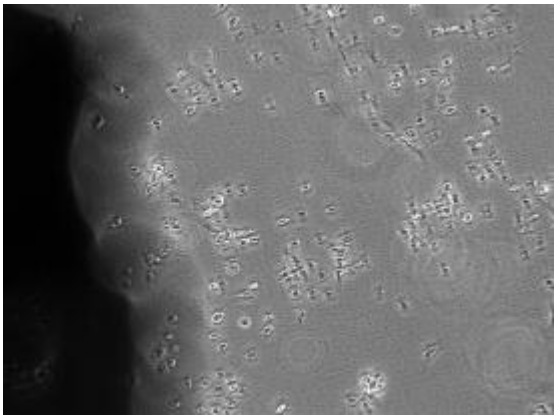
2B.



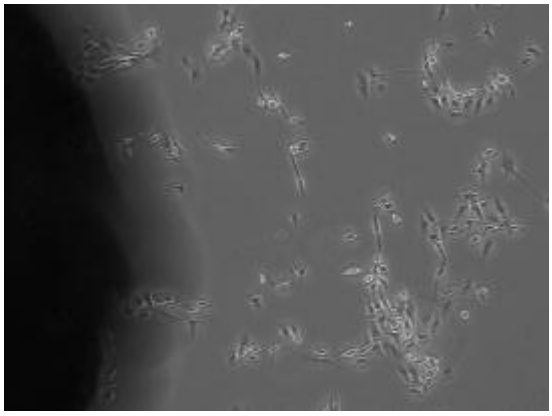
3A.



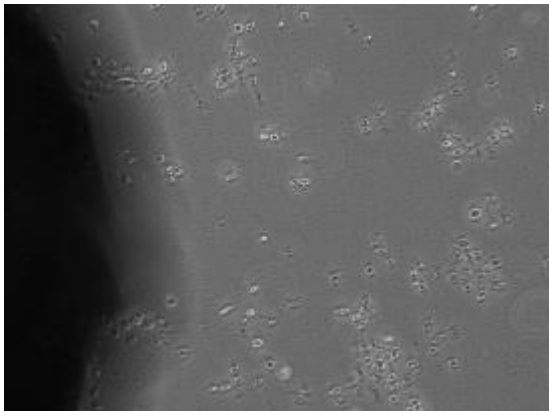
3B.



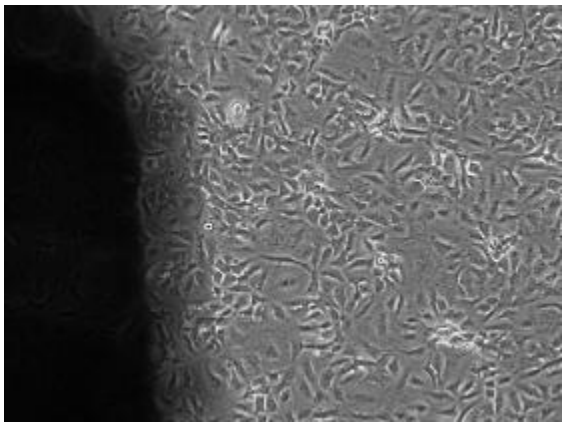
4A.



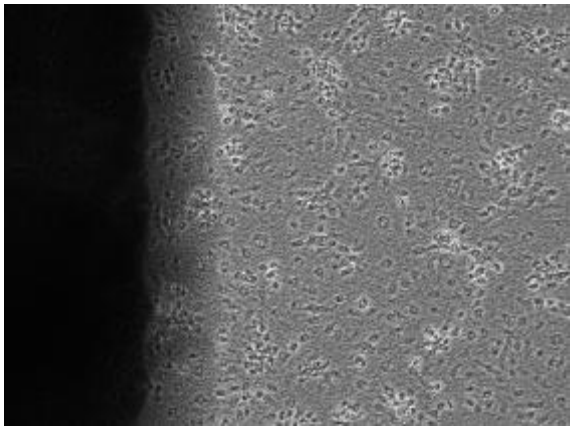
4B.



5A.

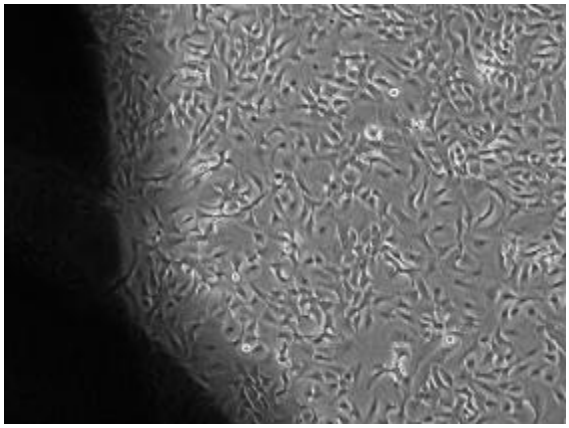


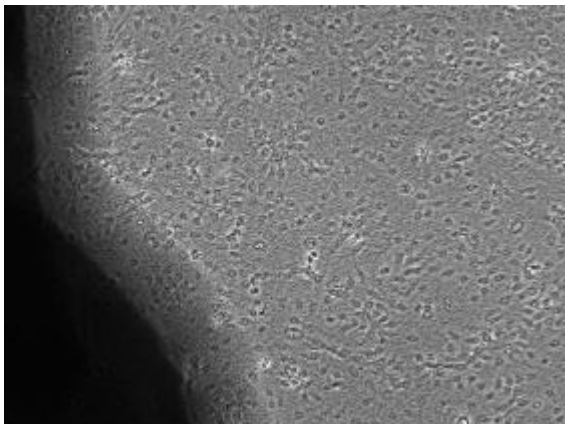
5B.



6A.

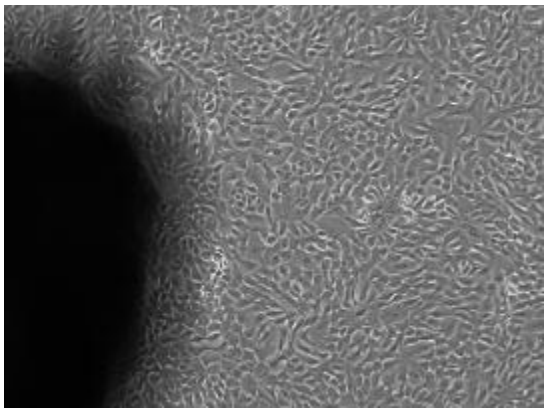
6B.



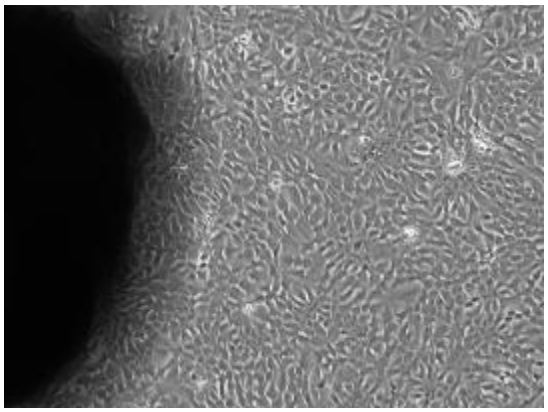


Appendix G. Photographs at 5x of Somatostatin Pre (A) and Post (B) Treatment

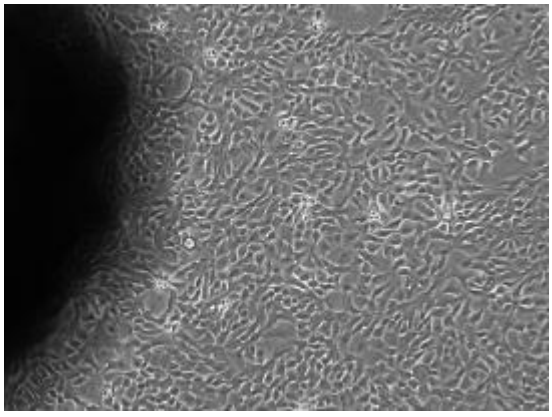
1A.



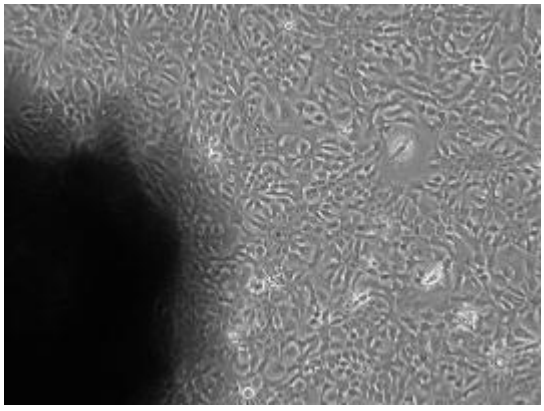
1B.



2A.

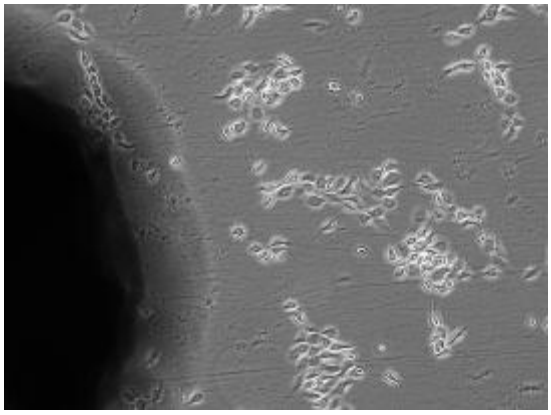


2B.

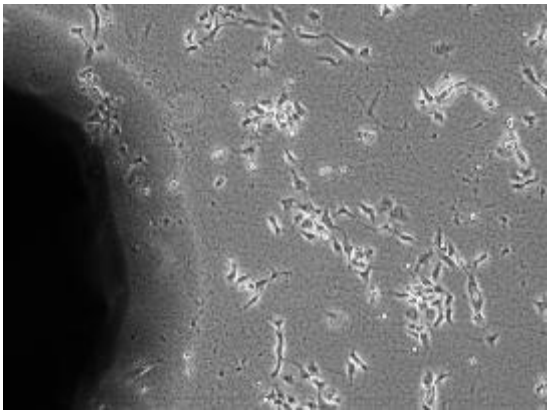


3A.

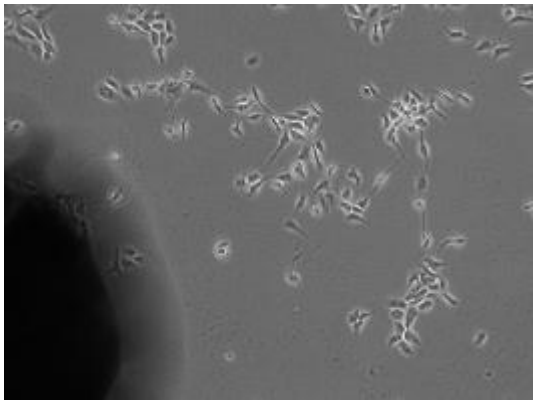
3B.



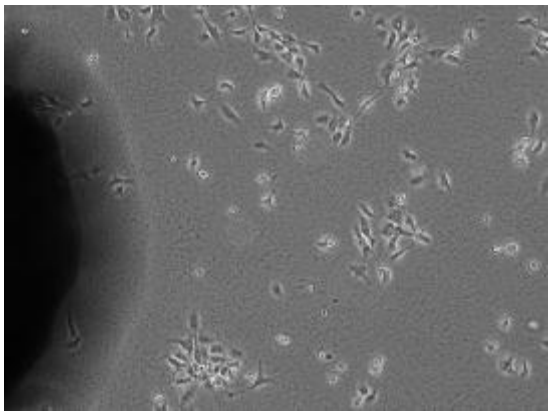
4A.



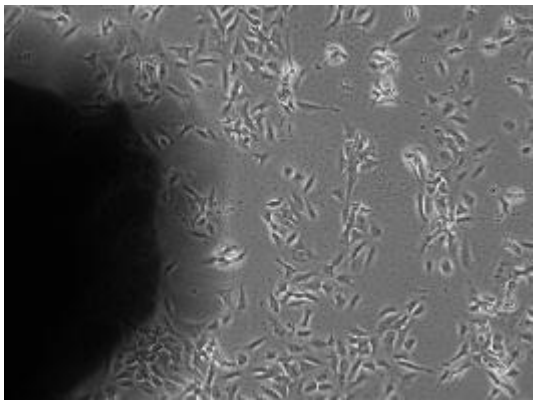
4B.



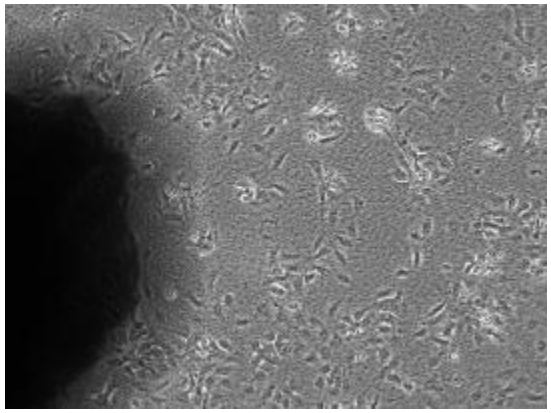
5A.



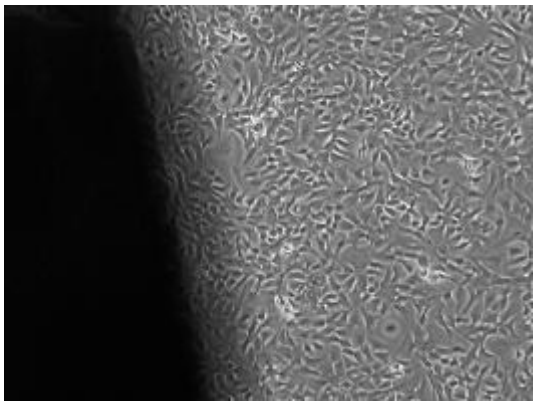
5B.

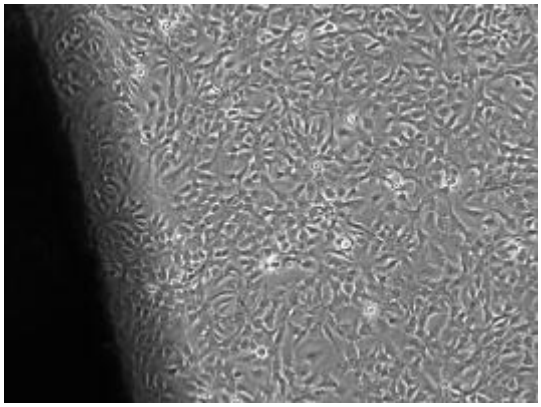


6A.



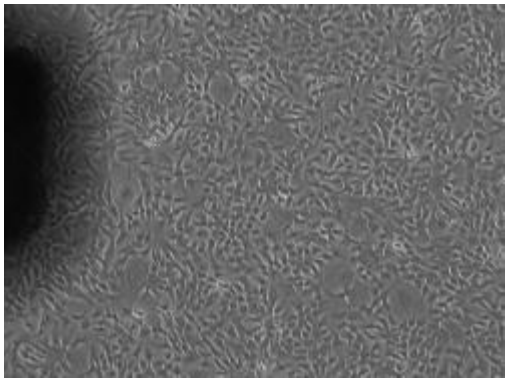
6B.



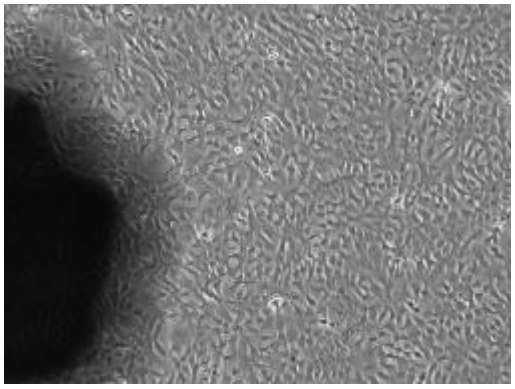


Appendix H. Photographs at 5x of TNF-β1 Pre (A) and Post (B) Treatment

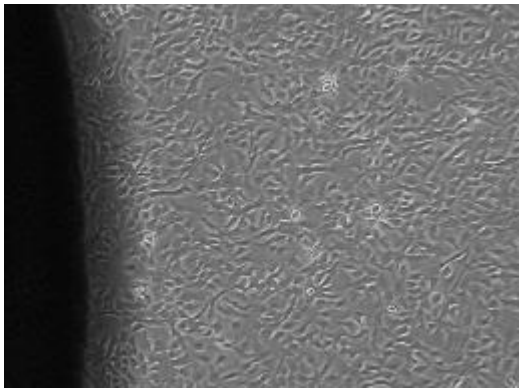
1A.



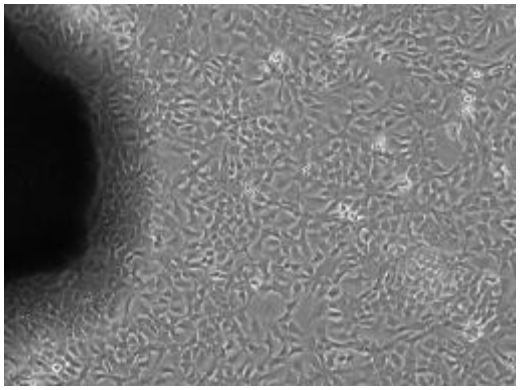
1B.



2A.

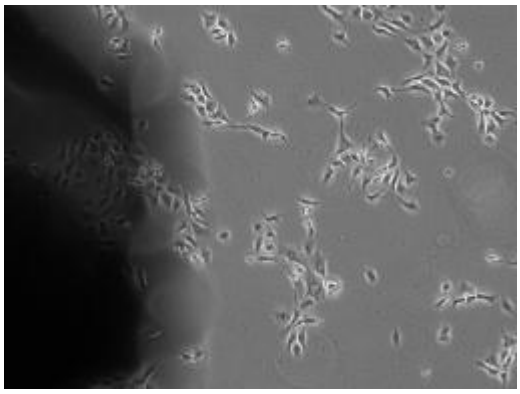


2B.

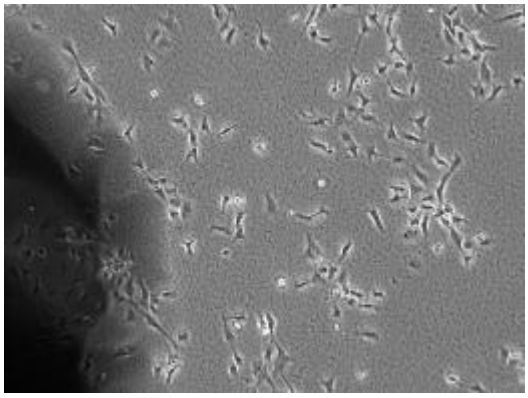


3A.

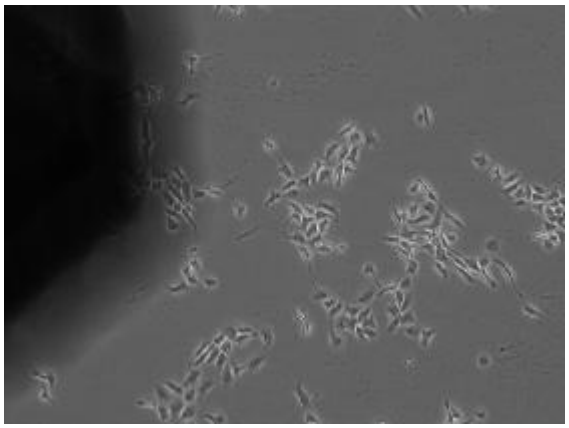
3B.



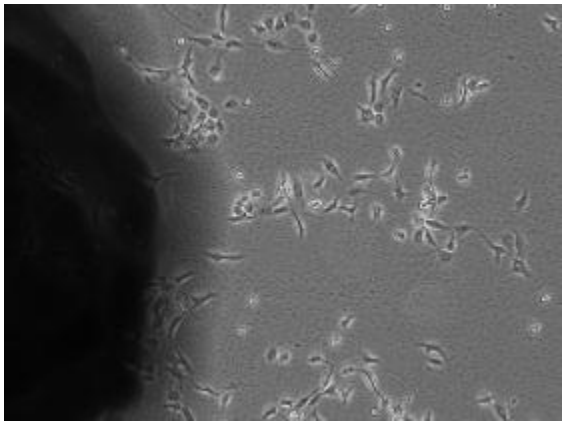
4A.



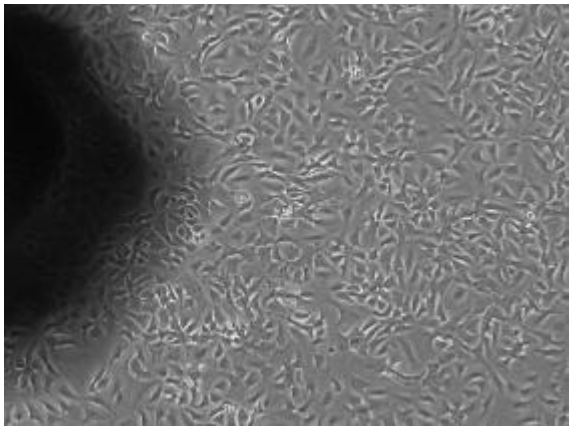
4B.



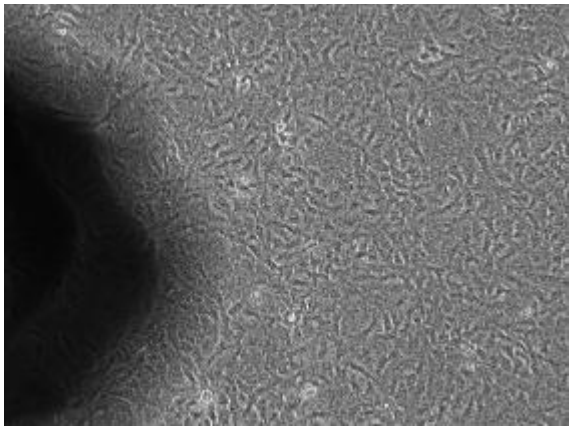
5A.



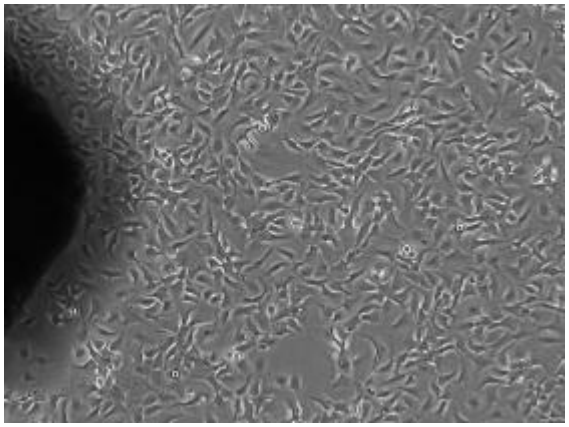
5B.

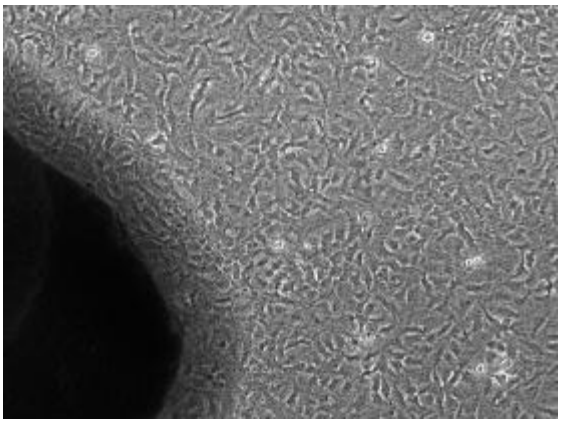


6A.



6B.





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